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NOTE CONCERNING THE IDENTITY OF VOLEMITOL AND α-SEDOHEPTITOL.

By F. B. La Forge and C. S. Hudson.

(Received for publication, June 18, 1928.)

Through the courtesy of Dr. F. Richter, editor of the Beilstein Supplements, it has been brought to our attention that an erroneous reference was given in our article on sedoheptose.[1] It was

CORRECTIONS.

On page iv, Vol. lxxviii, No. 1, June, 1928, second line from the bottom, following "1.75 gm. of glucose" read *per kilo (98.4 gm.)*.

On page 457, Vol. lxxviii, No. 2, July, 1928, Experiment 6, column headed "$(Lactate)_b$", for "Resting" read *1.2*.

Volemitol was discovered by Bourquelot[5] as a constituent of the rather rare mushroom *Lactarius volemus* Fr., m.p. 140–141° and $[\alpha]_D = +1.99°$ or $+2.40°$ in aqueous solution. This rotation is

[1] La Forge, F. B., and Hudson, C. S., *J. Biol. Chem.*, 1917, xxx, 61.
[2] Bougault, J., and Allard, G., *Compt. rend. Acad.*, 1902, cxxxv, 796.
[3] Bourquelot, E., *J. pharm. et chim.*, 1895, ii, 389.
[4] von Lippmann, E. O., Chemie der Zuckerarten, Brunswick, 1904, 2nd edition, 1002.
[5] Bourquelot, E., *Bull. soc. mycologique France*, 1889, v, 132–163.

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(Received for publication, June 18, 1928.)

Through the courtesy of Dr. F. Richter, editor of the Beilstein Supplements, it has been brought to our attention that an erroneous reference was given in our article on sedoheptose.[1] It was there stated that "the melting point of the benzal derivative of volemite is given by Bougault and Allard[2] as 90°, which does not agree with the melting point (200°) for the benzal compound of the heptitol [α-sedoheptitol]." The fact is, however, that Bougault and Allard did not describe such a compound of volemitol. It had been previously mentioned by Bourquelot[3] as a substance crystallizing in silky needles (no melting point stated), and he also described a crystalline ethyl acetal of volemitol melting at 190°. Von Lippmann's "Chemie der Zuckerarten"[4] recorded a m.p. of 90° for the benzal derivative of volemitol but it is quite apparent now that there is no authority for this statement and that it was erroneously repeated in our article. The data concerning volemitol and its derivatives that are recorded in several standard compilations contain so many erroneous statements that we have thought it advisable to record briefly here a correct record of the experimental data that have been published on the subject.

Volemitol was discovered by Bourquelot[5] as a constituent of the rather rare mushroom *Lactarius volemus* Fr., m.p. 140–141° and $[\alpha]_D = +1.99°$ or $+2.40°$ in aqueous solution. This rotation is

[1] La Forge, F. B., and Hudson, C. S., *J. Biol. Chem.*, 1917, xxx, 61.

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not affected by the addition of boric acid. In a later article Bourquelot[6] records that the addition of borax greatly augments the dextrorotation of volemitol, $[\alpha]_D$ being about +22° to +29° in an aqueous solution containing 1.85 gm. of borax per 25 cc. A crystalline acetate of volemitol (m.p. 119°, $[\alpha]_D$ = +19.15° in acetic acid), a crystalline ethyl acetal (m.p. 190°, levorotatory) and a crystalline benzal derivative (m.p. not recorded) are mentioned.

Fischer,[7] working with a 10 gm. sample of volemitol which Bourquelot sent him, found its m.p. 149–151° after four recrystallizations from alcohol and its $[\alpha]_D^{20}$ = +1.92° in 10 per cent aqueous solution. The oxidation of volemitol by bromine in alkaline solution yielded a reducing sugar which formed a crystalline phenylosazone and the analysis of this substance showed volemitol to be a heptitol.

Bougault and Allard[2] isolated crystalline volemitol from the roots and rhizomes of several species of *Primula* (*Primula grandiflora, elatior* Jacq. and *Primula officinalis* Jacq.) with a yield of about 15 gm. per kilo of dried plant. M.p. 154–155°, $[\alpha]_D$ = +2.65° in aqueous solution. Boric acid did not affect the rotation, but borax increased it to +20.83° in about 7 per cent borax solution. The ethyl acetal melted at 206° and showed $[\alpha]_D$ = −46° in chloroform solution. An acetate with a m.p. of 62° is mentioned but it is not clearly indicated whether the substance is crystalline. They conclude, however, that the acetate of m.p. 119° which Bourquelot described is not volemitol acetate but rather mannitol acetate and consider that Bourquelot's volemitol contained a small quantity of mannitol.

La Forge and Hudson[1] obtained α-sedoheptitol by the reduction of sedoheptose with sodium amalgam, m.p. 151–152°, $[\alpha]_D^{\infty}$ = +2.25° in aqueous solution, and $[\alpha]_D^{20}$ = +22.09° in about 6 per cent borax solution. The crystalline tribenzal derivative melted at 199–200°, but in a later article[8] this value was corrected to 225°.

[6] Bourquelot, E., *J. pharm. et chim.*, 1895, ii, 385–390. In this article Bourquelot refers to a publication by himself, "Sur la volémite, nouvelle matière sucrée retirée d'un champignon" in the Proces-verbaux de l'association française pour l'avancement des sciences, session de Pau, 1892, 183. This short publication is a summary of work more fully described in Bourquelot's other articles.

[7] Fischer, E., *Ber. chem. Ges.*, 1895, xxviii, 1973.

[8] La Forge, F. B., *J. Biol. Chem.*, 1920, xlii, 375.

La Forge[8] recrystallized a sample of volemitol furnished him by Bourquelot and found a m.p. of 151°, and this value was not changed when the material was mixed with α-sedoheptitol. He prepared the ethyl acetal of α-sedoheptitol and found a m.p. of 191–194° and $[\alpha]_D = -45°$ in chloroform solution. The agreement of these data with the results of Bourquelot (m.p. 190°) and of Bougault and Allard (m.p. 206°, $[\alpha]_D = -46°$) led him to conclude that volemitol and α-sedoheptitol are identical, and that the configurational formula of volemitol is in consequence that which he had proved for α-sedoheptitol; namely,

$$\begin{array}{c} \quad H \quad H \quad H \quad H \quad OH \\ CH_2OH \cdot C \cdot C \cdot C \cdot C \cdot C \cdot CH_2OH \\ OH \; OH \; OH \; OH \; H \end{array}$$

or its mirror image.

Finally, the data which show the identity of α-sedoheptitol with volemitol are here summarized.

	Volemitol.		α-Sedoheptitol.	
	M.p.	$[\alpha]_D$	M.p.	$[\alpha]_D$
	°C.	degrees	°C.	degrees
Alcohol.	151	+2.65 (Water.) +20.8 (Borax solution.)	151	+2.25 (Water.) +22.1 (Borax solution.)
Ethyl acetal.	(206) (190)	−46 ($CHCl_3$)	191–194	−45 ($CHCl_3$)

The alcohols when mixed show a m.p. of 151°.

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THE PRECIPITATION OF BLOOD CALCIUM BY LEAD.

By FRITZ BISCHOFF and L. C. MAXWELL.

(From the Chemical Laboratory of the Potter Metabolic Clinic and the Department of Cancer Research, Santa Barbara Cottage Hospital, Santa Barbara.)

(Received for publication, June 19, 1928.)

INTRODUCTION.

The treatment of inoperable cancer by the intravenous injection of suspensions of lead has stimulated interest in the fate of this metal in the blood stream. The original metallic colloid of Bell (1) is very sensitive to oxygen, and by *in vitro* experiments Brooks (2) and Bischoff and Blatherwick (3) showed that there was little likelihood of any of the metallic lead reaching the cancer as such. Aub and Reznikoff (4) had shown that the phosphates and carbonates were the only two of nine constituents normally found in blood which would neutralize the damaging effect of ionic lead to red cells, the phosphate ions being many times as effective as the carbonate. Fairhall and Shaw (5) found that the lead deposited in bones in lead poisoning is present as phosphate and not as carbonate or albuminate, and from a critical study of the solubilities of lead carbonate, dilead phosphate, and trilead phosphate, concluded that trilead phosphate was the form in which lead was transported in the blood in lead poisoning. Bischoff (3, 6) prepared various colloidal suspensions of lead with the inorganic constituents of the blood and found from rabbit tests that colloidal metallic lead, ionic lead and lead hydroxide were about equally damaging to the red blood cells. The oxycarbonate was less damaging and the phosphate without effect. Ionic lead, buffered with serum or red blood cells containing sufficient inorganic phosphorus to convert all the lead to phosphate, was also found to be non-toxic. The colloidal phosphate prepared for the rabbit experiments was then tried clinically by Ullmann (7), Pulford (8), Soiland, Costolow, and Meland (9), and others.

Brooks (2), experimenting with the Bell metallic colloid and ionic lead, found that when these substances were added to serum, the amount of phosphorus precipitated from the serum (determined by analyses of ultrafiltrates), was higher than the theoretical amount required to convert the lead to the triphosphate and corresponded more nearly to the diphosphate. Brooks explained his results by assuming a delayed equilibrium in which the dilead phosphate was first formed. At the end of 70 hours, the ratio of lead to phosphorus still corresponded to the dilead phosphate. Bischoff

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(6) showed that when ionic lead is added to the blood, the amount circulating after 2 hours is nil. On the basis of Brooks' interpretation, there would be little chance of any trilead phosphate being formed from the metallic colloid by the time the lead reached the tumor, and the experimental evidence for the clinical use of the trilead phosphate would be materially weakened. From his lead:phosphorus ratios, Brooks (2) concluded that in a phosphate buffer, lead acetate and colloidal metallic lead formed the triphosphate; in Ringer's solution the former formed the triphosphate and the latter the diphosphate, and in blood serum they both formed the diphosphate. These results suggested to us that calcium might be thrown out with the lead in the cases where the ratio pointed to the diphosphate, and that no dilead phosphate but a precipitation of lead and calcium triphosphates or a double triphosphate salt took place. Brooks' analytical results would be in complete harmony with this explanation.

$$3PbX_2 + 2Na_2HPO_4 = Pb_3(PO_4)_2 + 4NaX + 2HX$$
$$2PbX_2 + CaX_2 + 2Na_2HPO_4 = Pb_2Ca(PO_4)_2 + 4NaX + 2HX$$

His results with Ringer's solution made this explanation seem likely, since this solution becomes highly supersaturated with calcium when the pH is alkaline. In the case of lead acetate in which he obtained the ratio for the tri-salt the Ringer's solution would become more acid. For the colloidal lead, where the ratio for the di-salt was obtained, it would become more alkaline.

$$3Pb(Ac)_2 + Na_2HPO_4 + NaH_2PO_4 = Pb_3(PO_4)_2 + 3NaAc + 3HAc$$
$$3Pb(OH)_2 + Na_2HPO_4 + NaH_2PO_4 = Pb_3(PO_4)_2 + 3NaOH + 3H_2O$$

Object.

The experiments recorded in this paper were taken up with the idea of establishing whether or not calcium was involved in the reaction of lead ions with blood serum. A few preliminary experiments showed that ultrafiltrable calcium was thrown out of solution. The amounts of phosphorus and calcium removed from solution were greater than is required for the above equations. In order to interpret these results in the light of recent investigations as to the state of calcium in the blood, complete data for pH, CO_2-combining power, inorganic phosphorus, and diffusible and non-diffusible calcium were taken.

Analytical Methods.

Inorganic phosphorus was determined by the Fiske-Subbarow modification of the methods of Bell and Doisy and Briggs. The maximum experimental error was within 5 per cent. Calcium

was determined by the Clark-Collip modification of the Kramer-Tisdall method. Duplicate analyses checked well within 0.3 mg. of Ca. CO_2-combining power was determined by the Van Slyke gasometric method and in some experiments by the manometric method. Analyses of our ultrafiltrates showed a variation of 5 per cent in CO_2-combining power as compared with the value obtained for the original serums. The pH was determined colorimetrically with Clark and Lubs' indicators and standard buffer solutions. Values were duplicated within 0.05 pH in the range of pH 6.8 to 7.4 and within 0.1 pH for the other values given.

Experiments with Ringer's Solution and Phosphate Buffer.

The Ringer's solution contained 8 gm. of NaCl, 0.2 gm. of KCl, 0.22 gm. of $CaCl_2$, 2.22 gm. of $NaHCO_3$, and 0.24 gm. of KH_2PO_4 in 900 cc. The pH was adjusted to 7.2 by adding dilute HCl. To 180 cc. of this solution were added 20 cc. of a mixture containing 42 mg. of $PbCl_2$ and 2.3 cc. of 0.1 N NaOH. The pH after the addition of the basic lead solution shifted to 8.2. The mixture was stirred half an hour and filtered. 6.2 mg. of Ca and 4.0 mg. of P per 100 cc. were found in the filtrates. The original Ca and P on a 100 cc. basis were 8.4 and 6.0 mg. respectively. The amount of phosphorus removed from solution lies between the values required for the di- and triphosphates. There is not enough phosphorus removed from solution to convert both the lead and calcium to the triphosphate. The results of the experiment in Ringer's solution indicated that some calcium carbonate was also thrown down with the phosphate. The experiment was tried in a phosphate buffer solution containing calcium chloride. It is interesting to note that both this phosphate solution and Ringer's containing calcium chloride were supersaturated with respect to calcium phosphate. Thus a calcium chloride-phosphate solution was made by adding 0.24 gm. of KH_2PO_4, and 11 cc. of 0.1 N NaOH solution to 900 cc. of CO_2-free water at 60°. To this solution 0.22 gm. of $CaCl_2$ in 100 cc. of water was added. The mixture was placed in a thermostat for 1 hour at 37° and a slight precipitate filtered off. The analysis of the filtrate gave 5.3 mg. of P and 5.0 mg. of Ca per 100 cc. The pH was 6.7. If the ionic strength is taken as 0.008 and the

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apparent solubility product at that ionic strength as pK 31.0 from Holt, La Mer, and Chown's (10) data for the solubility product of tricalcium phosphate, at 37°:

$$(Ca)^3\ (PO_4^{\equiv})^2 = \text{(solubility product)}$$
$$X^3\ ((1.71 \times 10^{-3})\ (6.7 \times 10^{-7}))^2 = 1.0 \times 10^{-31}$$

where 1.71×10^{-3} is the concentration of phosphorus in mols per liter, and 6.7×10^{-7} is the fraction present as $PO_4^{\equiv}$ at the given pH.

$X = 9.2 \times 10^{-5}$, mols Ca for saturation
1.25×10^{-3}, mols Ca present

The filtrates remained perfectly clear for days. To two 100 cc. portions of such a phosphate-calcium chloride solution were added 50 mg. of lead acetate and 50 mg. of lead acetate neutralized with NaOH, respectively. The original pH of the buffer solution was 7.3. Adding lead acetate changed the pH below 6.0. Adding the neutralized acetate took the pH to over 9.0. 2.9 mg. of P were removed in the lead acetate experiment (theory for the triphosphate 2.75 mg.). 4.4 mg. of P were removed in the basic lead experiment (see Table I). These experiments were performed at room temperature, 23°. The experiment with the phosphate buffer and lead acetate was repeated at 37° with the same results. An experiment with Ringer's solution under CO_2 tension was also performed at 37°. In the experiments with phosphate buffer a considerable shift in pH was brought about by the liberation of acetic acid, because the concentration of phosphate (about the same as in blood) was low. In Ringer's solution the relatively high concentration of bicarbonate lessened this effect. The results of this experiment were particularly interesting. An amount of phosphorus corresponding to trilead phosphate was thrown out of solution, confirming a similar experiment of Brooks; at the same time all the calcium down to a concentration of 0.2 mg. per 100 cc. was removed after standing 1 hour. After the solution had stood 30 hours the amount remaining was not titratable. In the analysis for calcium 5 times the volume of solution usually taken was used to increase the accuracy; this was necessary because of the small amounts of calcium remaining in solution. The experiment shows that at the pH of the

TABLE I.

Effect of Lead Ions upon Ringer's Solution, Calcium Chloride-Phosphate Solution, and Blood Serum.

Before lead

TABLE I.

Effect of Lead Ions upon Ringer's Solution, Calcium Chloride-Phosphate Solution, and Blood Serum.

Medium.	Before lead.					After lead.					Phosphorus calculated.				
	pH	Inorganic P.	Total Ca.	Dialyzable Ca.	Pb added.	pH	Inorganic P.	Combined CO_2.	Dialyzable Ca.	Removed.	Required for $Pb_3(PO_4)_2$.	Required for $Pb_3(PO_4)_2$, $Ca_3(PO_4)_2$.	Required for $PbHPO_4$, $Ca_3(PO_4)_2$.	Required for $PbHPO_4$, $CaHPO_4$.	$\frac{\text{Pb added}}{\text{Ca removed}}$.
		mg. per l.	*mg. per l.*	*mg. per l.*	*mg. per l.*		*mg. per l.*	*mg. per l.*	*mg. per l.*	*mg. per l.*	*mg. per l.*	*mg. per l.*	*mg. per l.*	*mg. per l.*	
Ringer's solution + $Pb(Ac)_2$	7.7	59		18.5	338	7.55	25	838	0.5	34	33.8	51.0	67.8	76.4	
" " + $Pb(OH)_2$	7.2	60		84	156	8.2	39		62	21	15.6	27.0	34.9	40.5	1.37
Phosphate-$CaCl_2$ + $Pb(Ac)_2$	7.3	54.5		79	275	<6.0	25.4		79	29	27.5				
" + "	6.7	53		50	275	4.9	27		50	26	27.5				∞
" + $Pb(OH)_2$	7.3	54.5		79	275	>9.0	6		(39)	48	27.5	(48.1)	(61.8)	(72.2)	∞
Serum.															1.33
Experiment I.	8.3	67	140	96	268	8.3	9.5 ?		57.5	57?	26.8	46.7	60	70	1.34
" II.	8.2	52	140	97	110	8.2	32	294	75	20	11.0	22.4	27.9	33.5	0.97
" III.	7.35	47	161	108	159	7.15	14	670	80	33	15.9	30.4	38.2	45.5	1.09
" IV.	7.3	71.5	145	105	198	7.25	36	520	70.4	35.5	19.8	37.7	47.5	56.4	1.09
" V.	7.55	88	112	90	259	7.45	34.9	1022	43	53	25.9	50.2	63.1	75.2	1.07
Serum and oxalate.															
Experiment VI.		80	12	8.6	520	7.1	28	700	1.3	52	48	52			
" VII.		50	32	10.4	394	6.95	9	773	2.1	41	35.3	39			

The figures in parentheses indicate calculated values.

blood, lead acetate throws out both the calcium and the phosphorus, but that the amount of phosphorus is not sufficient for the triphosphate of both the lead and the calcium. Some carbonate is undoubtedly removed at the same time (see Table I). The data will be discussed again in connection with the experiments with blood serum.

These experiments showed that the difference in results obtained by Brooks in a phosphate buffer and in Ringer's solution could be accounted for by precipitation of calcium, and did not indicate the formation of lead diphosphate. It will be noted that basic lead suspensions, prepared just before use, were taken instead of metallic colloidal lead solutions. This was done because colloidal lead solutions always contain appreciable amounts of lead carbonate (10 per cent) and since it has been shown that the colloidal lead is rapidly oxidized to lead hydroxide when added to a solution saturated with air.

Experiments with Blood Serum.

The apparatus described by Brooks (2) was used in the experiments with blood serum. The collodion bags were made from du Pont parlodion dissolved in the amounts of alcohol-ether recommended for surgical collodion. Filtration was conducted under a pressure of 200 to 300 mm. of Hg. A known volume of serum was introduced into the bag, and a sufficient quantity ultrafiltered to determine the pH, calcium, phosphorus, and bicarbonate, and to make a biuret test. In the first three experiments the lead acetate peptized in 2 cc. of serum was added to the serum in the bag. After thorough mixing, the ultrafiltration was continued, the first few cc. being discarded. In the fourth experiment, the lead acetate was not added to the serum remaining in the bag, but to a fresh sample of the same serum. In the fifth experiment the lead acetate was added directly to the total volume remaining in the bag. It was thought that the way in which the lead was added might affect the results. Alveolar air was used in the CO_2 tension experiments. The apparatus was first swept out with this gas mixture, which was also bubbled through the serum several minutes before the serum was introduced into the bag. A stream of the mixture was also kept bubbling through the serum while the lead acetate was being peptized and mixed with the serum. In

the first four experiments rabbit serum was used; in the fifth pig serum. The ratio of diffusible to total calcium for the four rabbit serums was fairly constant, varying from 67 to 72.5 per cent. The total calcium value is about 3 mg. higher in the rabbit serum than the value given for human serum. There was sometimes a slight difference between the inorganic P and always a difference between the bicarbonate values for the serum and its ultrafiltrates. The values for the ultrafiltrates were used in the calculations. The serums were preserved with phenol. One serum was used without the addition of a preservative.

The preliminary experiments with blood serum were performed without CO_2 tension. The experiments with phosphate buffer containing calcium chloride had shown that the precipitation of the calcium depended upon the pH of the medium, lead acetate, which produced a greater acidity, throwing down no calcium. Brooks had shown that in serum there was no difference in the phosphorus removed from solutions whether one used lead acetate or colloidal lead. Our experiments were confined to lead acetate. From the solubility product theorem, the calcium in the serum should become more supersaturated as the alkalinity is increased. With use of lead acetate this condition would not arise.

The results of our experiments showed that calcium was thrown out of solution whether one worked at a pH above 8 without CO_2 tension or at a pH of 7.4 with CO_2 tension. The amount of calcium thrown out of solution was equivalent to the amount of lead added. The amount of phosphorus removed from solution corresponded to the triphosphates of lead and calcium. It will be noted that the phosphorus removed from solution is higher than most of the values reported by Brooks and that the calcium is twice as high as would be required if one interpreted Brooks' findings on the basis of a mixed tricalcium lead phosphate instead of dilead phosphate. Brooks gives two values which are 10 per cent, and one which is 5 per cent higher than the theoretical value of his diphosphate. His other values are 5 to 10 per cent lower. In determining the phosphate value, the difference between two analytical values which may be in error by 5 per cent, is taken. The same applies to the calcium, for while the analytical values for calcium are probably more accurate than those for phosphorus, the difference between the calcium values is a smaller per cent

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than the difference between the phosphate values. The higher values for phosphorus that we obtained in our experiments may be due to a difference in technique. The constancy of the ratio for added lead to precipitated calcium is well within the analytical error.

The amount of acid liberated by the reaction of the lead acetate with the phosphate in the serum is not great enough to shift the pH more than 0.2. Thus in Experiment III, the magnitude of the concentration of the various compounds expressed in mols per liter, was as follows:

7.65×10^{-4} lead added.
6.45×10^{-4} acetic acid liberated.
9.08×10^{-4} sodium acetate liberated.
4.5×10^{-4} inorganic phosphorus left.
1.65×10^{-2} bicarbonate.

} Calculated from the M_2HPO_4: MH_2PO_4 ratio at the initial pH.

The acetic acid liberated is equivalent to but 5 per cent of the bicarbonate content.

TABLE II.

Apparent Solubility Products for $CaCO_3$ and $Ca_3(PO_4)_2$ in Serum after Lead.

The concentrations are expressed in mols per liter.

pH	[Ca] $\times 10^{-3}$	Total CO_2 $\times 10^{-2}$	$[CO_3^=]$ $\times 10^{-5}$	P $\times 10^{-3}$	$[PO_4^{\equiv}]$ $\times 10^{-9}$	$[Ca] \times [CO_3]$ $\times 10^{-6}$	pK $CaCO_3$	$[Ca]^3 \times [PO_4]^2$ $\times 10^{-25}$	pK $Ca_3(PO_4)_2$
8.3	1.43			0.36	23.2			15.7	23.8
8.2	1.88	0.67	20.8	1.03	61.8	39.0	6.41	236	22.6
7.15	2.00	1.5	3.23	0.45	1.51	6.46	7.19	0.182	25.7
7.25	1.76	1.27	3.38	1.16	5.39	5.47	7.23	1.58	24.8
7.45	1.08	2.43	10.4	1.13	9.9	11.2	6.95	1.23	24.9

Mechanism of the Calcium Precipitation.

The work of Holt, La Mer, and Chown and Hastings, Murray, and Sendroy (11) has shown that the amount of calcium in blood serum greatly exceeds the amount which should be present, calculated from the solubility product of calcium carbonate or calcium phosphate as determined in a salt mixture of the same ionic strength as serum. Whether this calcium is in a supersaturated, a colloidal, or a non-ionizable state does not appear to be definitely established. In order to interpret our data in the

light of the solubility product conception, the apparent solubility product for calcium carbonate and calcium phosphate has been calculated from the analytical data of our serum ultrafiltrates (see Table II). Since the calcium in these filtrates has passed through a membrane which holds back proteins, it is obviously not in a colloidal state nor bound in a non-diffusible form to protein. The CO_3 ion and PO_4 ion concentrations are calculated from the tables furnished by Hastings, Murray, and Sendroy (11) for carbonate, and Holt, La Mer, and Chown (10) for phosphate. In the calculation of Hastings, Murray, and Sendroy, the ionization constant is corrected for ionic strength. This was not done by Holt *et al.* Since our data are calculated for comparative and not absolute values, this difference is not essential to the interpretation of the results.

One might suppose that when calcium phosphate is thrown out of serum by the addition of lead acetate, an equilibrium would be established between the calcium phosphate in the solid and solution phases, and a constant solubility product would be obtained. Our data show that this is not the case (see Table II). The apparent solubility product for $Ca_3(PO_4)_2$ varies in each experiment. An error of 10 per cent in calcium and 0.1 in pH would affect the $pK_{s.p.}Ca_3(PO_4)_2$ by 0.35 and the $pK_{s.p.}CaCO_3$ by 0.16. In these experiments, the ultrafiltrates obtained 1 hour and 24 hours after the addition of lead were analyzed with no change in the results, so that equilibrium had apparently been reached. Since the solubility of calcium salts is not appreciably affected by the changes of temperature, we felt justified in comparing our solubility products, which were obtained at 23°, with those of Hastings, Murray, and Sendroy and Holt, La Mer, and Chown at 37°. Hastings, Murray, and Sendroy give pK $CaCO_3$ as 6.40 in serum and 7.40 in salt solutions of the same ionic strength. Our pK values at pH 7.15 to 8.3 for $CaCO_3$ equal or exceed 6.40, so that at equilibrium, the solution is not supersaturated with respect to calcium carbonate. Holt, La Mer, and Chown give 26.0 as the pK $Ca_3(PO_4)_2$ in serum and 27.2 in salt solutions of the same ionic strength. All our pK values are below this figure. The serums are still supersaturated with respect to calcium phosphate after the lead has been added. Sendroy and Hastings (12) have shown that when serum is shaken with solid $CaCO_3$, no precipita-

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tion of calcium takes place, but that when $Ca_3(PO_4)_2$ is used, the calcium content of the serum decreases rapidly, equilibrium being reached in 5 minutes. Our results would therefore tend to show that the calcium was not precipitated as $Ca_3(PO_4)_2$ but as a mixed lead-calcium phosphate. The constant ratio of lead added to calcium precipitated could better be explained on this basis. If the precipitation of this double lead-calcium phosphate is not governed by the solubility product for tricalcium phosphate, and if the double salt is more insoluble than calcium phosphate, the calcium should be precipitated from a solution which was not saturated with respect to calcium phosphate. In order to test this experimentally, over 90 per cent of the calcium was removed from a serum with sodium oxalate. The calcium remaining in solution did not exceed the amount required for saturation. Upon addition of lead acetate to this solution, all the calcium was precipitated.

EXPERIMENTAL.

Precipitation of Calcium beyond the Saturation Point of Tricalcium Phosphate.

It was found that when the amount of oxalate theoretically required to convert the calcium to oxalate was added to the serum, 10 per cent of the total calcium was not precipitated and a little less than 1 mg. per 100 cc. was still ultrafiltrable. Thus 14 mg. of sodium oxalate were added to 30 cc. of serum which analyzed 13 mg. of calcium per 100 cc. After the mixture had stood overnight the calcium oxalate was centrifuged off; 1.2 mg. per 100 cc. of calcium remained, of which 0.86 mg. per 100 cc. was ultrafiltrable. To 37 cc. of another serum were added 15.4 mg. of sodium oxalate. The total calcium was reduced from 13.2 mg. per 100 cc. to 3.2 mg. per 100 cc. The ultrafiltrable calcium was 1.04 mg. per 100 cc. Lead acetate was added to these serums under CO_2 tension, as in the other serum experiments, and the calcium determined in the ultrafiltrates. In order to increase the accuracy of the calcium determinations, double the volume usually taken was used in the analytical determination. All the calcium, within the limits of the determination, was thrown out by the lead.

If pK 26.0 is taken as the solubility product of calcium phosphate in serum,

$$X^3 ((0.94 \times 10^{-3})(2.8 \times 10^{-6}))^2 = 10^{-26}$$
$$X = 1.1 \times 10^{-3}$$

or 4.4 mg. of Ca per 100 cc. required for saturation in Experiment VI with serum.

$$X^3 ((0.29 \times 10^{-3})(1.7 \times 10^{-6}))^2 = 10^{-26}$$
$$X = 3.5 \times 10^{-3}$$

or 14 mg. of Ca per 100 cc. required for saturation in Experiment VII with serum.

In these calculations, 0.94×10^{-3} and 0.29×10^{-3} are the concentration of P in mols per liter after the addition of the lead, and 2.8×10^{-6} and 1.7×10^{-6} are the factors which give the amount of PO_4 ion at the pH of the solution. It will be noted (Table I) that in the experiments recorded above the phosphorus removed from solution corresponds very nearly to the amount required for trilead phosphate. In the calculations given in Table I, we have corrected the results for the amount of lead which would be precipitated as oxalate. This amount is small and does not materially affect the final result.

An examination of the data for the experiment in which lead acetate was added to a Ringer's solution with a low calcium concentration, shows that the calcium has also been removed below the saturation point for either calcium carbonate or calcium phosphate. As this experiment was performed at 37° and the solid and liquid phases were in contact for 30 hours, a direct comparison with the data of Holt, La Mer, and Chown and Hastings, Murray, and Sendroy may be made. Thus:

$X (1.9 \times 10^{-2}) (5.5 \times 10^{-3}) = 3.9 \times 10^{-5}$
$X = 3.8 \times 10^{-4}$ mols of Ca required for saturation of $CaCO_3$.
$X^3 ((8.1 \times 10^{-4}) (1 \times 10^{-5}))^2 = 6.3 \times 10^{-28}$
$X = 2.1 \times 10^{-4}$ mols of Ca required for saturation of $Ca_3(PO_4)_2$.

These figures are equivalent to 1.52 and 0.84 mg. of Ca per 100 cc. of solution. The amounts found were 0.2 mg. after 1 hour and a trace after 30 hours. In this experiment there must have been some carbonate thrown down as well as phosphate. (The data

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show that carbonate was also thrown down when Ringer's solution was treated with lead hydroxide.) The significant fact is that calcium may be precipitated by lead below the saturation point in salt solution as well as in serum. An analogous example is the precipitation of radium with barium. The interpretation of this phenomenon by Lind (13) in Taylor's "A Treatise on Physical Chemistry" is quoted:

"The co-precipitation of radium and barium presents some highly interesting features. It seems anomalous that radium should be precipitated together with barium at a concentration far below that corresponding to the solubility of pure radium sulphate (about $\frac{1}{100}$ that of $BaSO_4$). This action is exactly that to be expected if radium and barium were truly isotopic, but they have, of course, atomic numbers widely different, and can readily be separated by fractional crystallization of the halides. Moreover, since they are not isotopic, one would expect the theoretically unstable condition established by the co-precipitation to revert to equilibrium by the return of radium into solution. Such is not the case. On the other hand, the degree of co-precipitation is not so rigid as with true isotopes."

We are not inclined to regard the calcium precipitation as one involving specific adsorption, because of the large amount of calcium removed from solution. It would, indeed, be unusual for a precipitate to adsorb a molecular equivalent of another salt. Fairhall (14) has worked out a method for the quantitative determination of lead in urine, in which the calcium is precipitated as the phosphate in ammoniacal solution with the quantitative entrainment of lead. The mechanism of this reaction is readily explained on the basis of our findings. It would appear that the action of the parathyroid hormone, which is as specific for lead as for calcium, is also in harmony with the double lead-calcium salt conception.

SUMMARY.

Lead acetate added to serum with or without CO_2 tension precipitates a molecular equivalent of ultrafiltrable calcium and an amount of inorganic phosphorus required for the formation of trilead and tricalcium phosphate. Since the amount of calcium thrown out is independent of the pH (6.9 to 8.3) and of the concen-

tration of phosphate, carbonate, and calcium ions, the reaction is not immediately concerned with the solubility product of calcium carbonate or calcium phosphate. The reaction is a specific lead effect.

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LACTIC ACID AND CARBOHYDRATE IN SEA URCHIN EGGS UNDER AEROBIC AND ANAEROBIC CONDITIONS.

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It has long since been established by Loeb (1) that unfertilized sea urchin eggs can exist for considerable periods of time when deprived of oxygen mechanically, or, when their oxidative processes are inhibited by the addition of cyanide to their medium. In the presence of potassium cyanide, as was shown by Loeb (1) as well as by the investigations of Warburg (2) and Meyerhof (3), the rate of aerobic respiration of the cells is greatly reduced or almost completely arrested. Such eggs will respire normally and will be capable of normal fertilization and subsequent development upon the addition of oxygen, or upon the removal of the cyanide by washing. This indicates clearly that the unfertilized eggs must possess an anaerobic metabolic process for the maintenance of life.

In an attempt to elucidate this phenomenon the analogy with the more recent findings or the anaerobic metabolism of tumor cells by Warburg (4), of yeast and other cells by Meyerhof (5) as well as of muscle cells deprived of sufficient oxygen, studied by Meyerhof (5) and by Hill and his school (6), inevitably presented itself. In all of the above investigations it was found that part of the energy required for the life processes was furnished by the anaerobic conversion of glucose to lactic acid. That lactic acid may be produced in sea urchin eggs was surmised by Loeb ((1), p. 31).

It was therefore decided to determine the lactic acid production in unfertilized sea urchin eggs under aerobic and anaerobic conditions.

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EXPERIMENTAL.

Mature unfertilized eggs of *Arbacia punctulata* were obtained daily at the height of the season at Woods Hole (July and August). After removal of the ovaries from the females they were suspended in sea water, strained through cheese-cloth, and the eggs washed by suspension and sedimentation in three changes of sea water. The washed eggs were then suspended in a definite small volume of sea water, rapidly stirred, and 1.00 cc. samples removed for nitrogen analyses. The pipette used for this purpose was the same throughout the reported experiments. It was provided with a wide open tip to permit a rapid removal of the sample before sedimentation occurred. The nitrogen determination was carried out as follows: The 1.00 cc. sample was blown out into a 50 cc. Erlenmeyer flask and 1 cc. of concentrated sulfuric acid added followed by 1 to 2 cc. of water. On slight warming the eggs dissolved almost completely. The solution was transferred with washings to a 25 or 50 cc. volumetric flask and the contents made up to the mark with distilled water. An aliquot of the resulting, slightly turbid, solution was used for analysis by the micro Kjeldahl method of Koch and McMeekin (7). Direct Nesslerization could not be made on the digested material because of turbidity which invariably developed, and distillation or aeration was used to recover the ammonia. Subsequent Nesslerization yielded clear and easily readable solutions. For the sake of uniformity all of the analytic results are expressed in terms of mg. of lactic acid (or glucose) per 1 gm. of egg protein; the approximate factor of gm. of $N_2 \times 6.25$ = gm. of protein was used. A similar method for the expression of analytic results on sea urchin eggs in terms of nitrogen was used by Meyerhof (3). This is believed to furnish a simpler basis for quantitative results than dry weight or the number of eggs.

Experiments with Potassium Cyanide.

The heavy suspension of the eggs in sea water described above was divided into two equal parts by volume. The same quantity of sea water was added to each, but the sea water added to one of these contained sufficient KCN to make its final concentration 0.02 per cent (approximately 0.003 M). The control and the

cyanide-treated egg suspensions were allowed to stand at room temperature (18–22°) for variable periods of time, 1 to 6 hours. If no large excess of sea water had been previously added, the suspension was triturated directly with the protein-precipitating reagent. In those cases where large amounts of sea water had been used, the eggs were sedimented by centrifugalization, and the supernatant fluid decanted and evaporated on a water bath to a conveniently small volume after the addition of a small amount of $NaHCO_3$ to render the solution distinctly alkaline. The sedimented eggs were triturated with the precipitating re-

TABLE I.

Effect of KCN on Lactic Acid Content of Unfertilized Eggs.

Experiment No.	Mg. lactic acid per 1 gm. egg protein.		Per cent increase.	Duration of experiment.
	Control eggs.	KCN eggs.		
				hrs.
1	3.27	5.60	72	4
2*	2.00	3.85	92	1
3*	2.05	4.20	105	6
4	2.80	4.71	68	2
5	2.95	7.14	142	2
6	4.39	5.14	17	2
7	5.13	8.64	69	6
8	2.44	6.13	151	1.5
Average....	3.14	5.68	81	

* Experiments 2 and 3 were begun simultaneously with eggs taken from one mixed suspension.

agents while the concentrated supernatant fluid was treated with the same protein-precipitating reagents and the lactic acid was determined separately in the filtrates. A corresponding amount of KCN was added to the suspension of control eggs, immediately before the addition of the precipitating reagents, in order to insure the strict comparability of the two solutions in the subsequent chemical manipulations. The results shown in Tables I and II express the figures for the total suspensions. The removal of the protein was attended with certain difficulties. After considerable experimentation it was found that the Schenk method of precipitation with HCl and $HgCl_2$, described by Cori (8), gave the

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best results. The filtrates, freed of mercury by H_2S, were treated with $CuSO_4$ and lime for the removal of interfering substances, as recommended by Clausen (9), and lactic acid determinations were carried out by the Friedemann, Cotonio, and Shaffer modification (10) of Clausen's method. The results are shown in Table I. A relatively large increase in lactic acid production in unfertilized eggs under the influence of KCN was found in every experiment.

Experiments with Fertilized Eggs.

In these experiments the preliminary treatment of the eggs was the same as described above, except that special care was taken to select fully mature eggs which did not show any cytolysis. For this purpose the eggs from each ovary were examined separately and a sample was subjected to a preliminary fertilization test and only such eggs were taken as showed at least 60 per cent membrane formation after 10 minutes. The suspension of eggs was again divided into two equal parts, one of which served as a control, while to the other freshly obtained sperm was added. Both suspensions were allowed to stand in a water bath at 20–21° in shallow dishes which were frequently and gently agitated to insure a plentiful supply of oxygen. Samples were examined under the microscope and the experiment terminated when about 90 per cent of the eggs had reached the two or four (or more) cell state of division. This period was usually 1 to 2 hours. Three experiments were terminated as soon as membrane formation had begun (after 7 to 12 minutes). In each case a corresponding amount of the same sperm was added to the control eggs immediately before precipitation of the protein.

It will be seen from Table II that while in each of the six fertilization experiments in which the embryo division was permitted to proceed to the two or four cell stage or beyond there was a definite increase in the lactic acid. This increase however is not nearly as great as in the case of the KCN-treated, unfertilized eggs. This can be easily explained by the enormously greater rate of oxidation proceeding in the dividing eggs as compared with that in the unfertilized eggs under the effect of KCN. The increase of the lactic acid in the eggs in which only membrane formation was allowed to take place was of the same order of magnitude as

in the divided eggs in two cases, while in one case an actual decrease was observed. The number of these latter experiments was too limited, however, to enable one to draw from them any definite conclusion.

Carbohydrate of Arbacia Eggs.

The question naturally arose as to the origin of the lactic acid. Meyerhof (3) in 1911 searching for the energy-yielding substance in the oxidative processes of the eggs of *Strongylocentratus lividus*,

TABLE II.

Effect of Fertilization and Cell Division on Lactic Acid Content of Eggs.

Experiment No.	Mg. lactic acid per 1 gm. egg protein.		Per cent increase.	Duration.	Remarks.
	Control eggs.	Fertilized eggs.			
				min.	
1	2.80	3.50	25	88	96 per cent fertilization, 4–8 cells.
2	2.95	3.31	14	95	70 " " " 4–8 "
3	3.56	3.90	10	122	90 " " " 4–8 "
4	1.37	1.56	14	130	96 " " " 2–8 "
5	2.35	3.53	50	95	97 " " " 4 "
6	3.25	3.58	10	73	98 " " " 2–4 "
Average.	2.71	3.23	19		
7	3.05	3.73	+22	12	100 per cent membranes formed, no cell division.
8	3.00	3.25	+8	7	
9	3.82	3.40	−11	7	

could not demonstrate the presence of any carbohydrate (glycogen, or copper-reducing substances) in these eggs. Nor could he demonstrate any manifestations of the cleavage of protein during the oxidative processes. He did find, however, large amounts of fat, and he was forced to regard the fat as the substance oxidized.

It is of interest to note that Mathews (11) found in the eggs of the starfish (*Asterias forbesii*) large amounts of a phosphatide which yielded on prolonged hydrolysis with acid 10 per cent of a reducing sugar which he could not definitely identify as glucose. Mathews informed us that he had found carbohydrate-containing lipids also in *Arbacia* eggs.

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Myers (12) reported the presence of 61 mg. of sugar per 100 cc. of "blood" in the Pacific sea urchin, *Strongylocentratus francescames.*

The present writers made a number of exhaustive attempts in search of free reducing sugars in the eggs of *Arbacia.* Even when as many eggs as those obtained from 50 sea urchins were used, not a trace of copper-reducing material could be found in concentrated protein-free extracts. In the attempts to isolate glycogen from the eggs by the Pflüger method of extraction with concentrated alkali, small amounts of material were obtained yielding an equivocal iodine test, and uncertain reduction tests after hydrolysis of this material with acid. Mr. K. Blanchard,[1] however, working later with dried eggs collected during the same summer at Woods Hole, found that if the eggs were previously

TABLE III.

Total Carbohydrate Content of Unfertilized Eggs.

Experiment No.	Mg. glucose per 1 gm. egg protein.	Duration of hydrolysis.
		hrs.
1	43	1
2	50	2
3	48	4
4	44	5
5	64	5

defatted, small amounts of glycogen could be obtained by Pflüger's method, which yielded on acid hydrolysis glucose, as shown by reduction tests and the melting point of the osazone.

In view of the uncertain results obtained by us in the search for glycogen it was decided to subject the eggs directly to acid hydrolysis in order to see whether combined carbohydrate was present in any form whatever. For this purpose the procedure described by Cori (13) was employed. Suspensions of eggs were hydrolyzed, with 0.5 to 1.0 N HCl in the water bath for 1 to 6 hours, the protein cleavage products removed with mercuric acetate, and the mercury removed with H_2S. In some instances colloidal iron was used instead of the mercury salt. The filtrates

[1] Personal communication.

so obtained showed in every case strong reduction with Fehling's and Benedict's reagents. When concentrated, good fermentation with yeast was produced and typical glucosazone crystals obtained.

In Table III some quantitative results are shown in terms of mg. of glucose per gm. of egg protein. The analyses were carried out with Folin's new sensitive sugar reagent (14) and with the use of Lloyd's reagent and permutit for the removal of interfering substances as recommended by Folin for urine.

It will be seen from Table III that the maximum amount of sugar is obtained already at the end of 1 hour's hydrolysis with acid.

DISCUSSION AND SUMMARY.

It has been shown in this investigation that mature unfertilized eggs of *Arbacia punctulata* produce lactic acid in the course of their normal aerobic metabolism. The concentration of lactic acid increases notably under the condition of anaerobic metabolism, in the presence of KCN. The concentration of lactic acid increases to a smaller extent after fertilization and in the early stages of cell division.

The reported results obviously furnish no information concerning the *rate* of formation of lactic acid under the aerobic and anaerobic conditions. It is possible that a greater concentration of lactic acid was found in the eggs treated with KCN, because under these conditions the oxidative processes, including the oxidation of lactic acid, are greatly retarded. Analogously the smaller increase in lactic acid in fertilized and actively dividing eggs may be due to the accelerated rate of oxidations in such eggs, causing the oxidative loss of some of the lactic acid.

The presence of free sugar could not be demonstrated in *Arbacia* eggs. The presence of glycogen was not definitely established by the present writers, but is reported to have been identified by another investigator in the same material. Hydrolysis of the unfertilized eggs with acid yielded readily a fermentable reducing sugar from which glucosazone was obtained. The amounts of total carbohydrate so found are more than ample to serve as the source of the observed lactic acid.

The anaerobic phase of metabolism of *Arbacia* eggs thus becomes quite analogous in its mechanism to that postulated for other tissues, such as tumors, yeasts, *etc.*

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Myers (12) reported the presence of 61 mg. of sugar per 100 cc. of "blood" in the Pacific sea urchin, *Strongylocentratus francescames.*

The present writers made a number of exhaustive attempts in search of free reducing sugars in the eggs of *Arbacia.* Even when as many eggs as those obtained from 50 sea urchins were used, not a trace of copper-reducing material could be found in concentrated protein-free extracts. In the attempts to isolate glycogen from the eggs by the Pflüger method of extraction with concentrated alkali, small amounts of material were obtained yielding an equivocal iodine test, and uncertain reduction tests after hydrolysis of this material with acid. Mr. K. Blanchard,[1] however, working later with dried eggs collected during the same summer at Woods Hole, found that if the eggs were previously

TABLE III.

Total Carbohydrate Content of Unfertilized Eggs.

Experiment No.	Mg. glucose per 1 gm. egg protein.	Duration of hydrolysis.
		hrs.
1	43	1
2	50	2
3	48	4
4	44	5
5	64	5

defatted, small amounts of glycogen could be obtained by Pflüger's method, which yielded on acid hydrolysis glucose, as shown by reduction tests and the melting point of the osazone.

In view of the uncertain results obtained by us in the search for glycogen it was decided to subject the eggs directly to acid hydrolysis in order to see whether combined carbohydrate was present in any form whatever. For this purpose the procedure described by Cori (13) was employed. Suspensions of eggs were hydrolyzed, with 0.5 to 1.0 N HCl in the water bath for 1 to 6 hours, the protein cleavage products removed with mercuric acetate, and the mercury removed with H_2S. In some instances colloidal iron was used instead of the mercury salt. The filtrates

[1] Personal communication.

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ON THE MECHANISM OF ENZYME ACTION.*

II. FURTHER EVIDENCE CONFIRMING THE OBSERVATIONS THAT ETHYLENE INCREASES THE PERMEABILITY OF CELLS AND ACTS AS A PROTECTOR.

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(Received for publication, August 25, 1927.)

Lavoisier (1) first pointed out that during the decomposition of the sugar molecule in the course of alcoholic fermentation, the molecule not only splits in two parts to form alcohol and carbon dioxide, but he also clearly indicated that alcoholic fermentation involves the phenomena of reduction and oxidation.

Later workers have shown that the phenomenon of fermentation is not a simple process but one involving the presence of a number of intermediate products which may well differ in ease of fermentability. Indeed it seems probable that the generally accepted chemical formula for certain of these intermediate products does not express the true state of the reactive substances but that an intermediate "transportation form" (2) may well be the reactive material. One of us (Nord (3))[1] has presented elsewhere a critical review of the current theories. Doubts concerning the exact mechanism of enzyme action (4) and the question as to whether intermediate products are acted upon under "biological" or "unphysiological" conditions (5), as well as the question as to whether the so called activators of enzymes really increase enzyme activity or whether they simply protect and prevent

* Preliminary communication, Nord, F. F., *J. Biol. Chem.*, 1927, lxxiv, p. lviii; *Protoplasma*, 1928, iv, No. 4.

[1] This paper on "Chemical Processes in Fermentations" contains a general discussion of the theories of chemical processes involved in fermentation and a more complete discussion of the literature than it is possible to include in the present paper.

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enzyme destruction, induced us to undertake the experiments reported in the present paper.

An investigation (6) of the physicochemical behavior of zymase solution, such as was prepared by Lebedew (7), led to the result that (*a*) it is possible to prepare zymase solutions which are capable of maintaining their biological activity. Data will be presented in this paper to show that these solutions maintained their full activity even after 65 days, although it has been generally assumed (8) that even at 0° the activity is lost within 48 hours. (*b*) It is not only possible to maintain the full activity of such a solution for practically an indefinite period, but it was shown that the enzyme solution, which behaves as a lyophile colloid, undergoes by means of appropriate peptization, a change of its surface forces, which is indicated by an increased surface tension and by decreased viscosity (6). Hence it was considered justifiable to conclude that the surface energy of the colloids concerned was increased, and this was reflected in the measurable increase of carbon dioxide production which was observed when the enzyme acted upon glucose.

After having reached, in certain cases, a production of 6.6 cc. of carbon dioxide per minute, the observation was made that this high fermentation activity was only maintained for a short time, the activity gradually falling to the average speed usually observed in enzymatic fermentation. This decrease could not have been due to an appreciable lack of fermentable sugar. It appears to be justifiable to interpret this observation as a further confirmation of an increased surface of the enzyme concerned. The increased surface is apparently much more sensitive toward the products of yeast metabolism. It might be assumed, therefore, that there are always enzymes present in a certain concentration, which are potentially capable of acting. However, since the reactivity of the enzyme is dependent upon its surface activity, it undergoes, immediately after the initial reaction, alterations which relatively decrease the velocity of the reaction, independently of the concentration of the reactant. In the course of the reaction, the proportion of damaged enzyme to active enzyme may be greatly increased. This conclusion indicates that there is a basis for the assumption that enzymes change their colloidal behavior even when bound to cells and under conditions where they may exert their activity.

Since we have, as yet, not been able to find a means whereby we can increase the surface of the acting enzymes within the living cell, we investigated the possibility of finding some method by which the original activity of the enzymes could be maintained, or by which the decrease of original activity could be delayed. Several considerations led to the idea that, independently of its reducibility, ethylene might be electrically charged on enzyme surfaces, in this way forming an adsorbed film, and since its opposed charges

$$\begin{array}{l} CH_2^+ \\ | \\ CH_2^- \end{array}$$

are neutralized (or in any event altered) in the condition of adsorption, it not only would not hinder the formation of the enzyme-substrate complex, but might act as a protector of the surface upon which it was adsorbed. Our experiments seem to have justified this view-point, since we not only could protect the increased surfaces which, as mentioned above, were produced by the appropriate peptization of the enzyme solution, but also found it possible to show in a striking manner that a protecting film could be formed even on enzyme surfaces which were within the cell.

The application of ethylene disclosed another very remarkable quality of this gas. It was possible to show through the action of ethylene on living single cells or cells in tissues, as for instance, tobacco leaves, that it increases the permeability when the cell systems are exposed to its influence. Since no definite data in this respect were available in the literature, it was necessary to be sure of this effect on permeability, since, for example, there has never been an agreement as to whether ether, which is also a very effective narcotic, causes an increase or a decrease of permeability, even though it inhibits cell division (9).

Through the fact that the permeability was increased, it was further possible to show that a yeast suspension, after having been exposed for a longer time to a slow current of ethylene gas, shows in the beginning a strongly increased carbon dioxide production, followed in the main course of the sugar fermentation by a decreased production as compared to the controls. If, on the

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other hand, an overcharging of the surface of the enzyme was avoided, the expected magnitude of the initial increased permeability was smaller, or even not noticeable, thus enabling the film to act as a protector, even during the fermentation of the first unit of glucose.

This observation led, on the one hand, to the connecting link between the alleged mechanism of enzymatic activity, and on the other hand, to the assumption, supported by this observation, that the physiological manner of acting of so called intermediate products in the biological form is fundamentally different from that in the unphysiological condition. It was Pfeiffer (10) who felt prompted to reemphasize recently that the association between enzymes and the substrate belongs in the group of true molecular combinations. If this be true, especially so far as zymases and fermentable sugars are concerned, it would be impossible to interpret the observations noted above, as well as those which are to follow.

Accordingly it was thought necessary to go a step further. In view of the well known fact that pyruvic acid exerts, probably because of its high degree of dissociation, an injurious effect on yeast cells, suspensions of yeast cells were allowed to act upon pyruvic acid solutions of different concentrations. It was not only possible to show that a 10 per cent suspension of yeast in a 1 per cent solution of pyruvic acid was capable of evolving practically the theoretical quantity of carbon dioxide within 26 hours, but the subsequent fermentation of 1 gm. of glucose in the original mashes showed, as expected, much less damaged enzyme in the case of the presence of a protecting film of ethylene than in its absence. It would be hard to understand how, in spite of the presence of a protecting adsorption film, a true molecular combination between enzyme and substrate could be assumed in our experiments, and it is much more plausible that these reactions also belong, in accordance with Gortner (11), to that group of reactions which may be regarded as governed by purely physical forces.

Mention has already been made of the effect of ethylene on single cells, and we believe that we have demonstrated that ethylene is not only capable of being charged as a protector on sensitive biological surfaces, but that due to adsorption it is able

to increase the permeability of cells. These observations were accordingly extended to cells in tissues, where, if the above observations were correct, the effect should be demonstrated perhaps even more strikingly. For this purpose we have investigated the effect on tobacco leaves, which, as we know from the investigations of Loew (12), contain a comparatively large quantity of catalase. The selected tobacco leaves, which were exposed to ethylene and then acted upon by a 6 per cent solution of hydrogen peroxide, evolved in fact a quantity of oxygen 15 to 20 per cent greater than did the leaves which were not exposed to ethylene.

These observations indicate furthermore that there occurs in the well known effect of the artificial ripening of fruits and vegetables (13) nothing less than an increase of the permeability of the cells, thus promoting the formation of the reactant-enzyme complex, and in this way advancing the hydrolysis of starch, from which are derived the sugar and other transformation products, and simultaneously, through the building up of the adsorbed protector film, enabling the enzymes to act for a prolonged time under conditions closer to ideal cases. In accordance with our considerations, there is no escaping the conclusion that the idiom "activation" is in this connection absolutely meaningless in its present use (14), especially since the phase of zymogens—if their existence according to our present uncertain knowledge may be considered as justified—might be regarded in our experiments as undoubtedly already having been passed.

EXPERIMENTAL.

Apparatus and Material.

The apparatus used for the fermentation experiments is the same as that described in a previous paper (6). The apparatus used in the catalase experiments consisted of a 150 cc., large mouthed, extraction flask, fitted with a 2-hole rubber stopper, a dropping funnel entering through one hole, and through the other a tube leading to a 3-way stop-cock, of which the two branches entered two burettes inverted in a dish of water. By applying suction to the burette tips, they can be easily refilled, and the

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use of the 3-way stop-cock permits the taking of consecutive readings without interference. The flask in which the hydrogen peroxide was acted upon by the catalase was kept in a constant temperature bath.

The ethylene used was a commercial product compressed in tanks, prepared for anesthetic purposes, and had a purity of 99.15 per cent.

The dextrose used was a chemically pure product. The yeasts were: (*a*) pure top yeast furnished by the Fleischmann Company, especially purified, and furnished at regular intervals, which insured its fresh condition for use in experiments; (*b*) bottom yeast supplied by the Minneapolis Brewing Company at regular intervals (after having been carefully washed and compressed).

Yeast juice was prepared from (*b*) in the following manner: The washed and carefully pressed yeast was dried under a vacuum of 34 mm. at 30° for 24 hours, in layers not exceeding 4 to 5 mm. in thickness. The dried yeast was then milled to grains of the average size of 1 to 2 mm. A 100 gm. portion of this yeast was introduced into 300 cc. of tap water, which had been previously saturated at 30° with carbon dioxide. The suspension was macerated in a mortar with a pestle until it was of a fairly uniform consistency, and was then submitted to maceration for 6 hours at 25–26°. In order to obtain a clear solution of the zymase which had partially come out of the cells, the juice was centrifuged for half an hour at approximately 4000 R.P.M. The solution was then carefully removed with a pipette from the sediment and submitted to a second centrifuging for the same length of time. The final juice had a tea color and was water-clear. Examination under the microscope indicated that it was absolutely free of cell particles, and it showed no spontaneous fermentation.

Zymin was also prepared from (*b*). 1000 gm. of yeast were suspended in 1000 cc. of water and most of the proteins of the plasma membrane were precipitated in an irreversible manner by adding, within 20 minutes, 6000 cc. of acetone. The product was allowed to stand 15 minutes in the acetone, then filtered, washed carefully with ether, and dried at 35° for 24 hours.

The pyruvic acid used was purified by distilling it *in vacuo* (at 8 mm. and 55°) in an atmosphere of carbon dioxide.

The hydrogen peroxide used was Merck's superoxol, containing about 30 per cent H_2O_2.

The tobacco leaves used were of the Blue Pryor strain.[2]

Method.

The solution or suspension used was always made up with a carbon dioxide-saturated tap water of an average initial pH of 6.0, containing 50 parts per million of calcium. The saturation and other preparatory operations were always carried out at the thermostat temperatures in order to avoid any influences due to contracting or expanding volumes.

The parallel experiment was conducted by bubbling ethylene through an opening of a 2 mm. tube, at a speed of 70 bubbles per minute, at a pressure of a 100 mm. water column for the time noted in each experiment.

The fermentations were carried out by the addition of 1 gm. (or amounts as stated in the experiments) of glucose. This gm. would furnish, if completely fermented, 282.4 cc. of carbon dioxide at 760 mm. and 30.5°. If pyruvic acid were completely fermented, 0.1 gm. would give 28.25 cc. of carbon dioxide under the same conditions. In most cases 20 cc. of the juice or of the yeast suspension, of which the type and concentration are noted in the discussion of the experiments, were used.

In all the fermentation experiments there was introduced quantitatively a second, and in some cases, a third gm. of glucose. These additions were made after the rates of the two fermenting mashes were approaching an identical magnitude.

During the main course of the fermentation, all readings were made at 1 minute intervals. In drawing the curves, however, for technical reasons, only greater intervals were plotted.

DISCUSSION.

1. Experiments on Yeast Juice.

Ethylene was bubbled through 20 cc. of freshly prepared juice for 30 minutes, and this solution was preserved overnight at

[2] The authors are indebted to Dr. James Johnson of the University of Wisconsin, for courtesies extended to them in supplying fresh tobacco leaves as required, and to Mr. R. B. Zimmermann, superintendent of the Minneapolis Brewing Company, for bottom yeast furnished.

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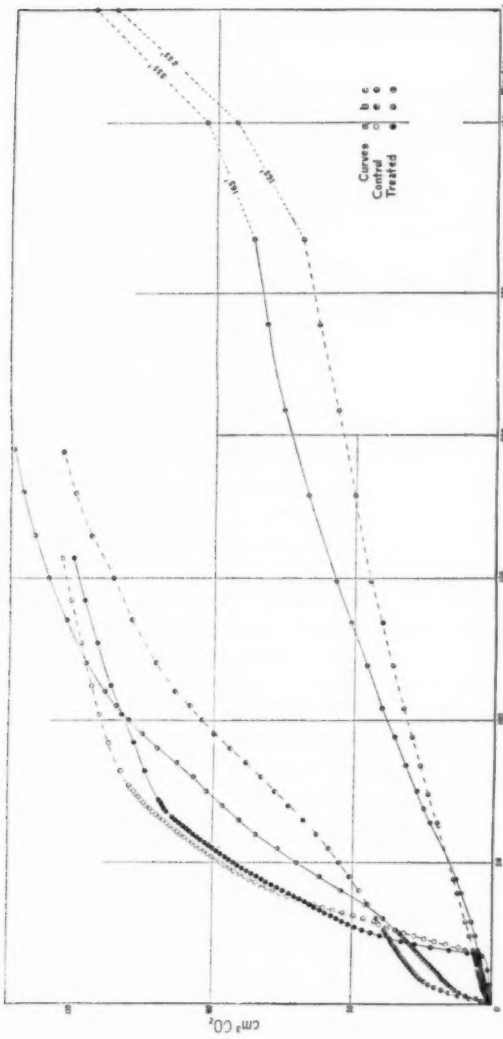

FIG. 1. (Experiment 1.) Fermentation of glucose with 20 cc. of yeast juice and with 20 cc. of yeast juice treated with ethylene. Curves (*a*) initial 1 gm. of glucose, curves (*b*) a second 1 gm. of glucose, curves (*c*) a third 1 gm. of glucose.

$-10°$. The control was prepared in a similar manner except for the gas treatment.

1 gm. of glucose was added to each solution just before commencing the measurements. The course of the fermentation is shown in curves (*a*), Fig. 1. After a period of 157 minutes a second gm. of glucose was added, and the course of the reaction, illustrated in curves (*b*) and (*c*), Fig. 1, shows the course of fermentation after the third gm. of sugar had been added. The difference in the rate of carbon dioxide produced is practically all in favor of the enzyme, treated with ethylene, especially during the course of the fermentation of the second and third gm. of glucose.

The juice used in the preceding experiment had been divided into two parts, and the results shown in Fig. 2 are those obtained 65 days after the preparation had been made.

Three different factors enter into this experiment. First, the once frozen juice was kept permanently at $-10°$ in order to avoid possible influences upon the physicochemical conditions of the enzymes due to repeated thawing. Second, in view of the well known antiseptic properties of toluene (15), 1 per cent of this was added to the mash ready for fermentation. Third, ethylene was bubbled through the treated enzyme solution for only 10 minutes.

It will be noted in comparing Figs. 1 and 2, that the rate of the fermentation shows not only no decrease when viewed from the standpoint that only 15 cc. of juice and 0.75 gm. of glucose were used in this experiment, but even indicates that the absolute rate of carbon dioxide production would have been increased if the data of observations had been calculated on the magnitude of the preceding experiments (20 cc. of juice + 1 gm. of glucose). Since it is generally assumed that the loss of the activity of a zymase solution is mainly due to the action of proteases on zymase, and that the temperature and time factors are very uncertain, the results of this experiment show conclusively that it was possible to reduce the protease action so that its influence was not detectable. In spite of the fact that our solution was absolutely free of cells, there was no assurance that it was absolutely free of proteases. The remarkable maintenance of the original activity appears therefore to be due to a very significant equalization of the action of proteases by the increased surface activity of lyophilic

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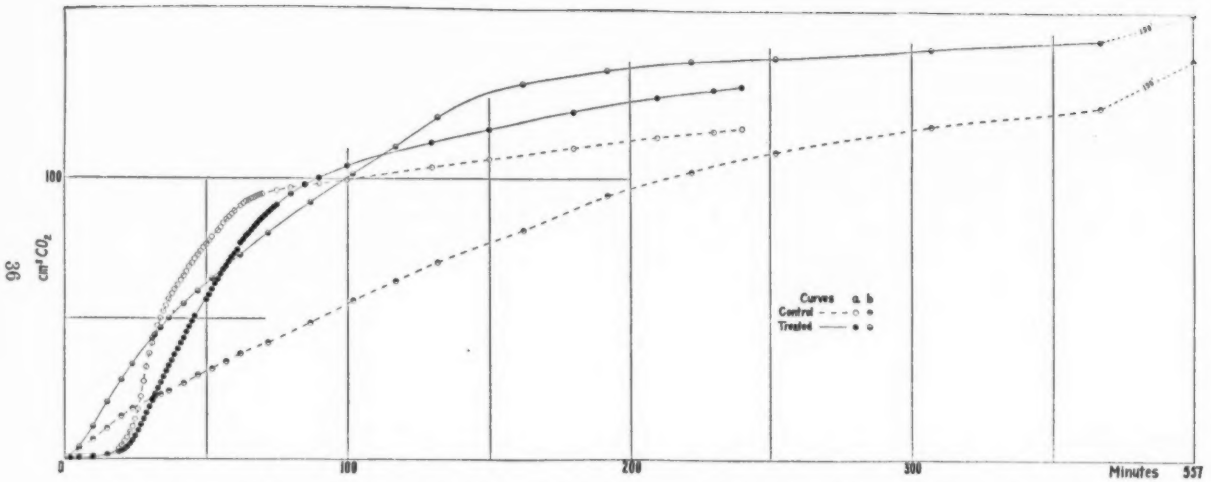

FIG. 2. The fermentation of glucose by 15 cc. of yeast juice preparations identical with those used in Experiment 1 (Fig. 1) after the juice had stood for 65 days at −10°. Curves (*a*) initial 0.75 gm. of glucose, curves (*b*) a second 0.75 gm. of glucose.

colloidal zymases. Therefore the conclusion might be drawn that in the complete absence of proteases, zymase solutions could be obtained which show not only practically indefinite keeping qualities, but are capable, in the beginning and within certain ranges, of showing a pronounced increase of their capacity to act on fermentable substrates.

The inspection of the curves indicates clearly the influence of a film adsorbed on the surface of the enzymes. This makes improbable two hypotheses; first, that in the case of fermentation, the enzyme-substrate complex may be considered as a true chemical combination, and second, that a film formed on the surface of a colloidal enzyme either by a gas, liquid, or solid compound, because of its reducibility, "activates" the processes. The main factor governing the effect is rather a physical one and depends on the capacity of the substances to be adsorbed, and in this way to interact between enzyme, substrate, or transformation products.

2. Experiments with Living Yeasts.

The preceding experiments led to the idea of applying our experiences in protecting enzyme surfaces to those in living yeast cells.

(*a*) *Bottom Yeast.*—The bottom yeast which served as a source for the zymase solutions in the above experiments was now used.

The purpose of these experiments was not only to show the possibility of the formation of a film on the surface of enzymes, bound on living cells, but to prove that it is a film, the formation of which is relatively independent of the period during which ethylene is bubbled through the preparation.

Three parallel experiments are shown in Figs. 3, 4, and 5, in each of which 20 cc. of a 10 per cent suspension acting upon each gm. of glucose were used. In the first experiment (Fig. 3) the enzyme surface was charged by bubbling ethylene for 30 minutes and kept at $+5°$ to $+8°$ for 12 hours. For the second (Fig. 4) the charging with ethylene was terminated after 10 minutes and the fermentation started subsequently. For the last experiment (Fig. 5) the ethylene gas was bubbled through the suspension for only 1 minute, followed by the immediate addition of sugar.

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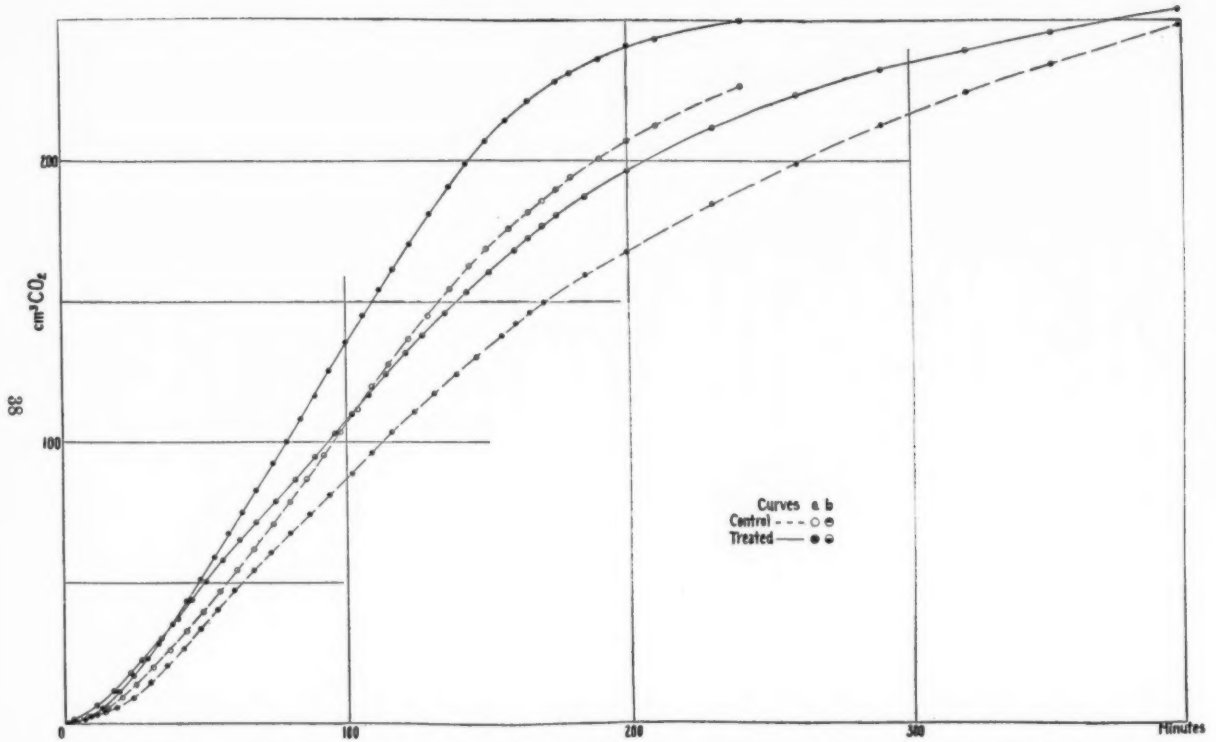

FIG. 3. The fermentation of glucose by a 10 per cent bottom yeast suspension and with a similar suspension treated for 30 minutes with ethylene. Curves (a) initial 1 gm. of glucose, curves (b) a second 1 gm. of glucose.

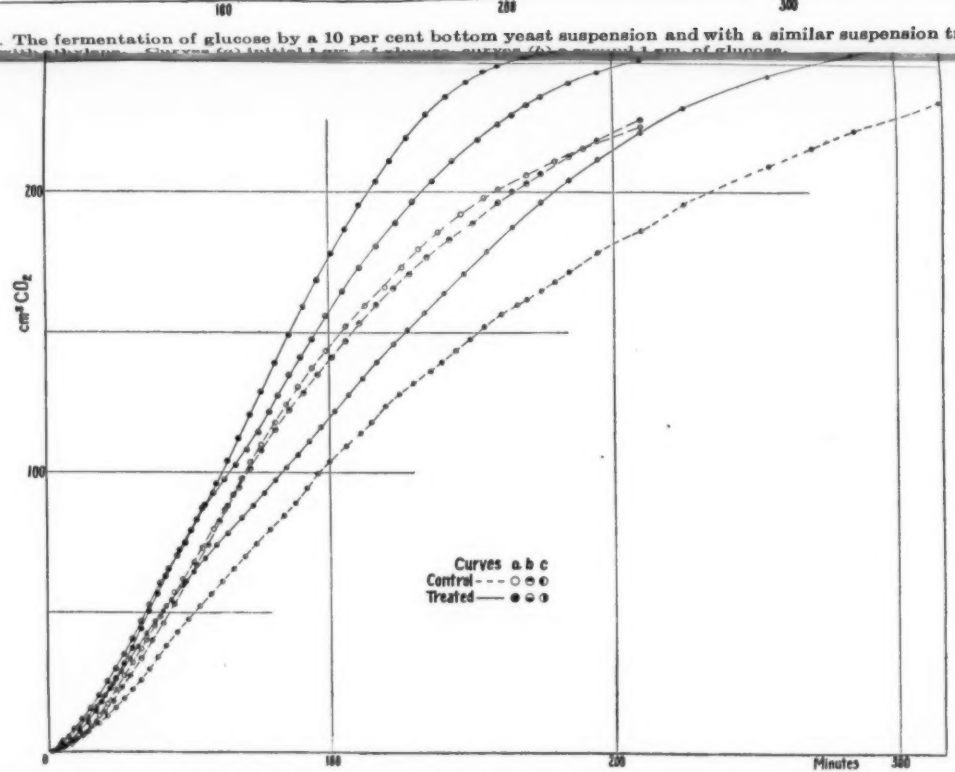

FIG. 4. The fermentation of glucose with a 10 per cent bottom yeast suspension and with a similar suspension treated for 10 minutes with ethylene. Curves (*a*) initial 1 gm. of glucose, curves (*b*) a second 1 gm. of glucose, curves (*c*) a third 1 gm. of glucose.

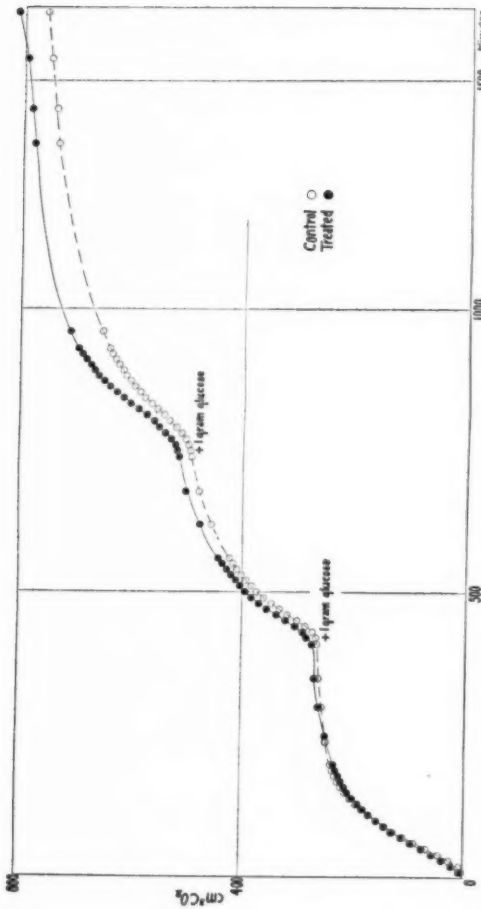

FIG. 5. The fermentation of glucose with a 10 per cent bottom yeast suspension and with a similar suspension treated for 1 minute with ethylene.

In all three experiments it is clearly demonstrated that the permeability of the treated yeast used was greatly increased. The decrease of the activity of the yeast enzymes, which is mainly due to the increasing concentration of the alcohol, was delayed not only during the fermentation of the first gm. of sugar, but more so during the course of the fermentation of the second and third gm. additions. In the case of the yeast treated with ethylene, this decrease is very much less than in the controls.

Comparison of the curves in Fig. 3 with those in Figs. 4 and 5 shows a lower fermentation rate, especially in the beginning, probably due to the fact that the enzyme surface was overcharged and the coating, according to Ramsden (16), passes gradually back into solution.

The fermentations represented by Fig. 5 picture the proceedings by a cumulative curve which indicates clearly the effect of the protecting surface against the transformation products, proving simultaneously that the ideal ratio of 1 between active and inactive enzymes falls much faster in those experiments where the enzyme is not protected.

(*b*) *Top Yeast.*—Similar experiments were carried out with a top yeast, whereby the general experimental conditions remained unchanged, except that the suspension used (Fig. 6) was in contact with ethylene 1 minute, and in the other case (Fig. 7) for 10 minutes.

However, in the case of fermentations represented by Fig. 7, a significant change was made in the procedure preceding the subsequent glucose additions. In order to make sure that *chemical* properties of the ethylene that may be present and dissolved in the mash were not responsible for the increasing delay of zymase inactivation, the liquid of the fermentation mash was removed by washing the yeast in the centrifuge after each finished fermentation, the washing being repeated three times with 70 to 80 cc. of water and the fermentation then being resumed on the addition of glucose to this washed suspension of original yeast.

The course of the reaction represented in both Figs. 6 and 7 confirms, without restrictions, the conclusions drawn from the fermentations carried out with bottom yeast.

(*c*) *Dough Fermentations with Top Yeast.*—Inasmuch as an increase of permeability in enzyme-containing cells is supposed

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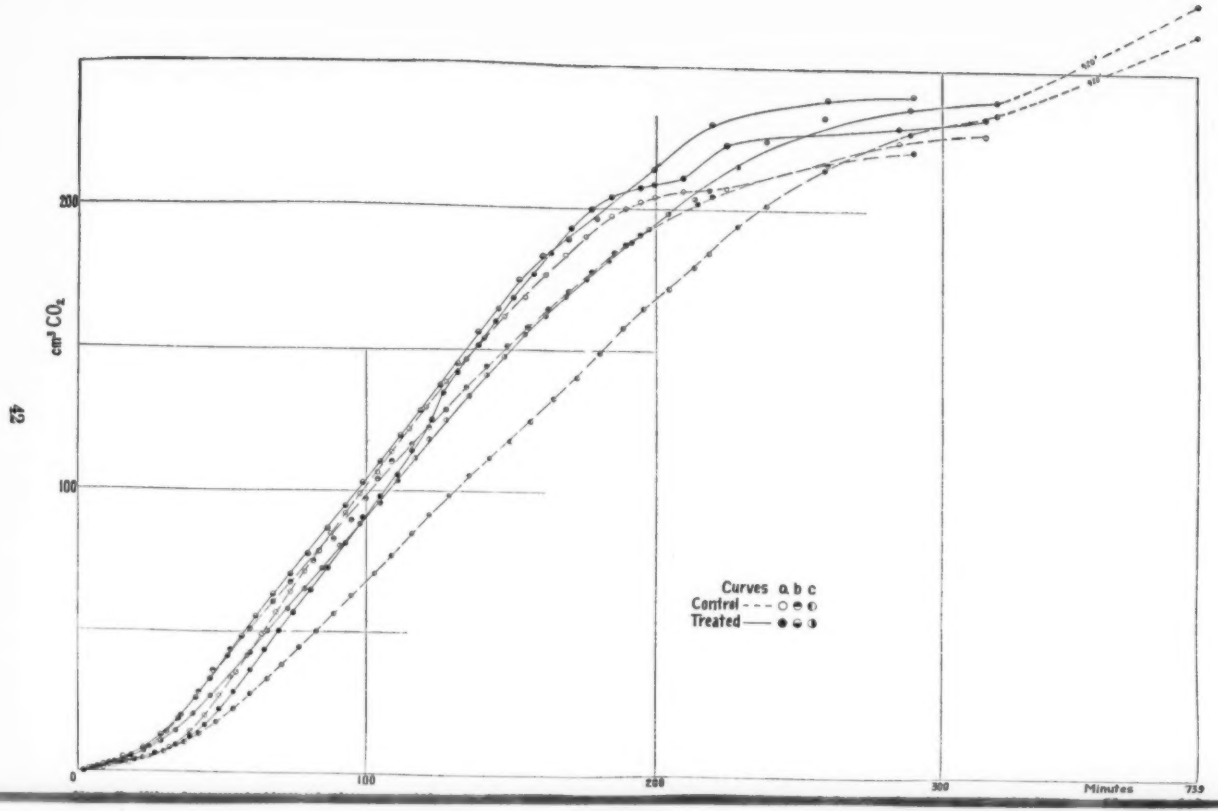
cm³ CO₂
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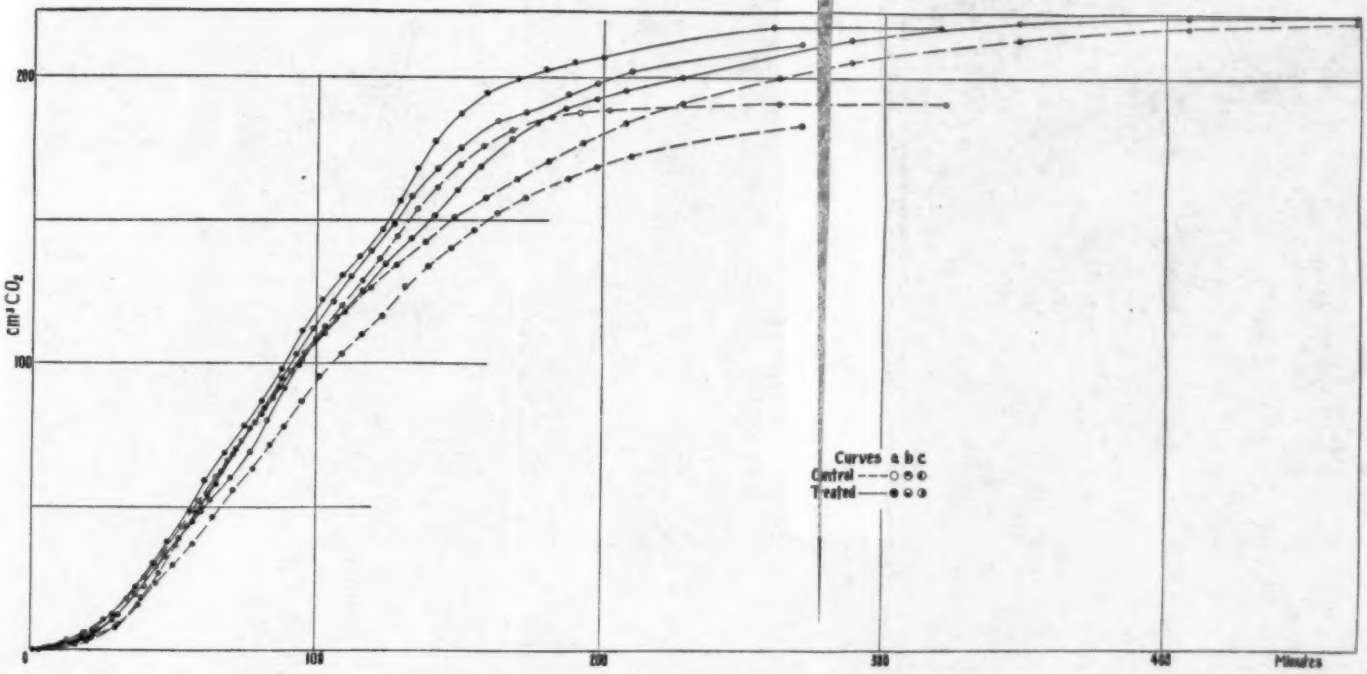

FIG. 7. The fermentation of glucose with a 10 per cent top yeast suspension and with a similar suspension treated for 10 minutes with ethylene. Curves (*a*) initial 1 gm. of glucose, curves (*b*) the washed yeast cells from the experiment represented by curves (*a*) and a second 1 gm. of glucose, curves (*c*) the washed yeast cells from the experiment represented by curves (*b*) and a third 1 gm. of glucose.

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to favor the formation of all enzyme-reactant complexes, it was thought advisable to carry out experiments in which a combination of enzymatic reactions takes place. A dough fermentation was considered the most suitable, since here a starch-gluten mass is acted upon by diastase on the one hand, and the transformation products of the diastatic activity as well as added sugar are acted upon by the invertase and zymases present on the other hand. In our experiments it was not possible to demonstrate that there was an augmentation of the diastase-starch reaction in the presence of ethylene. Such a possible influence of the ethylene could only take place in so far as free ethylene remained dissolved in the water in which the yeast was suspended.

A series of baking experiments[3] was carried out. Each batch of dough was made according to the following formula: 350 gm. of wheat flour, 10.5 gm. of sucrose, 5.25 gm. of salt, 10.5 gm. (or less) of yeast suspended in 50 cc. of water, 162.5 cc. of water, 9 gm. of compounded lard. This was fermented for 3 hours and 50 minutes. The procedure used is that which is considered as standard in baking tests.

The baking experiments carried out with 60 loaves demonstrated that there was a remarkable increase in the volume of those loaves which were prepared with the same quantity of yeast in which the cell permeability was increased by ethylene treatment. In good agreement with this fact, it was noted that the same loaf volume as the control was obtained by using 10 to 15 per cent less yeast, when the yeast had been previously treated with ethylene so that its permeability had been increased, or its enzymes were protected.

3. Experiments with Tobacco Leaf Catalase.

It was considered desirable to extend the investigations regarding the possible increase of permeability to cells in tissues. Tobacco leaves were accordingly chosen, since they contain, as already noted, a comparatively large quantity of catalase.

The experiments were carried out by shredding representative leaves of the Blue Pryor strain, and placing 5 gm. samples with 60 cc. of water in the reaction flask. The pulp, in curves (*a*)

[3] These tests were carried out by Mr. C. C. Fifield.

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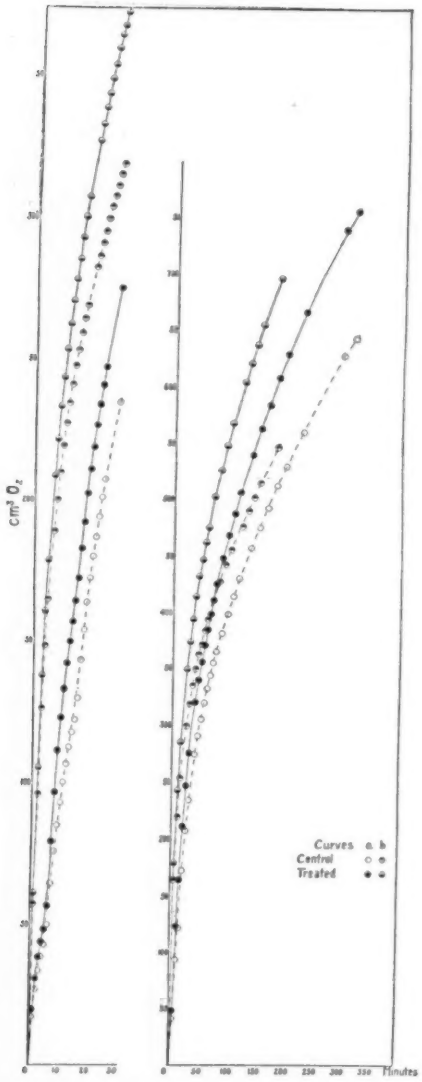

FIG. 8. The decomposition of H_2O_2 by catalase of tobacco leaf pulp and by tobacco leaf pulp treated with ethylene. The curves on the left show the cc. of O_2 evolved during the first 30 minutes of the reaction, those on the right the entire course of the reaction.

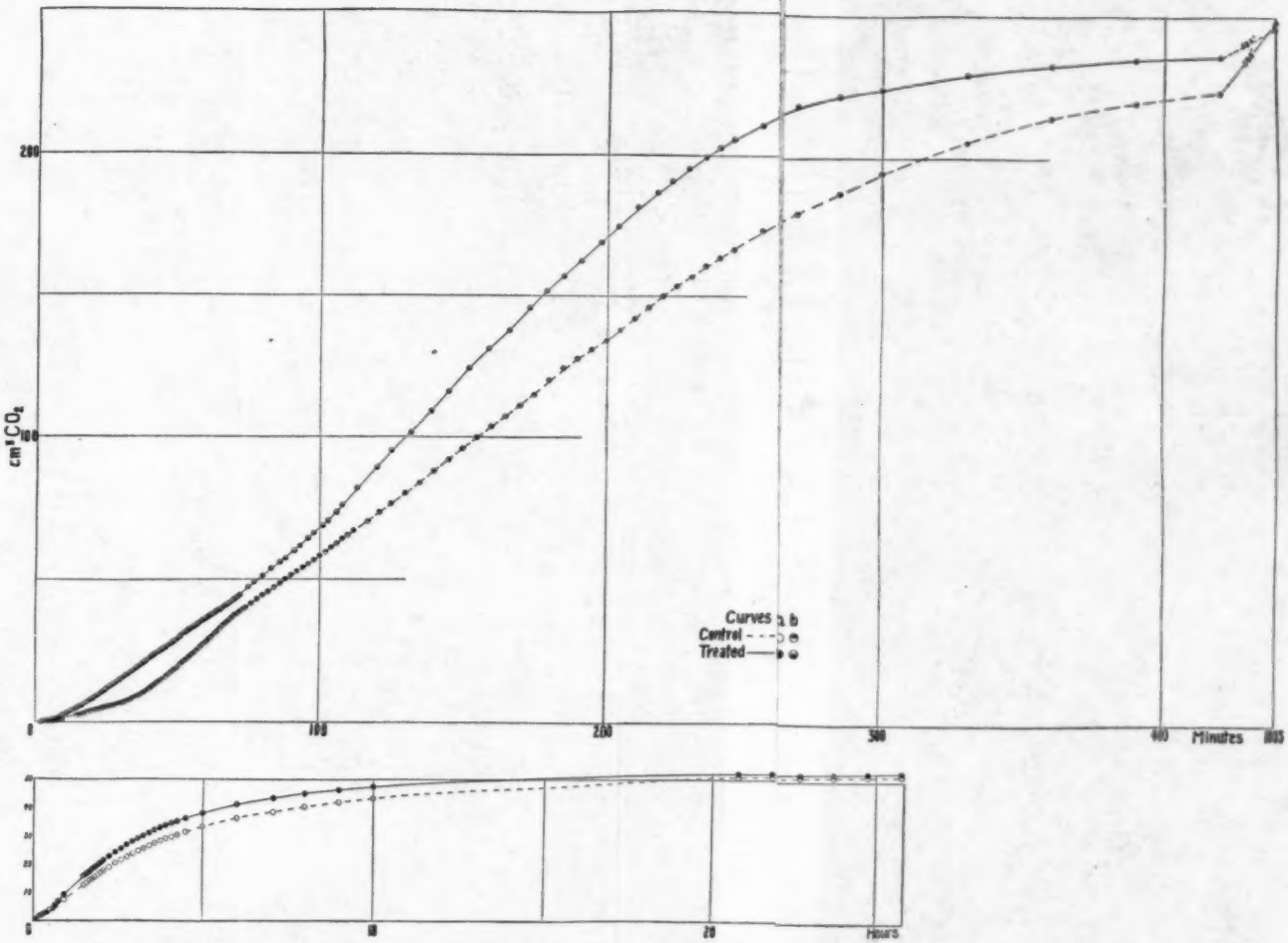

FIG. 9. The bottom curves (*a*) represent the fermentation of a 1 per cent pyruvic acid solution with a 10 per cent suspension of top yeast and with a similar suspension of top yeast treated with ethylene. The top curves (*b*) represent the fermentation of 1 gm. of glucose with the washed residual yeasts centrifuged after the completion of the above experiment.

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and (*b*), Fig. 8, was treated by bubbling ethylene gas through it for a few minutes in the usual manner.

It is easy to see from the curves that the treated leaves have a higher production of oxygen than the controls, and also that there is an increased oxygen production due to the increased permeability from the start. This may be seen by noting the curves on the left, which picture the course of the reaction during the period of the first 30 minutes. It can be easily recognized that an increased permeability occurred from the start, as the differences in the oxygen evolution during the entire course of the reaction are overwhelmingly in favor of the treated pulp.

4. Experiments with Living Yeast on Pyruvic Acid.

The experiments with pyruvic acid were carried out from two view-points. First, in spite of the fact that the earlier contradictory opinions (17) regarding the fermentability of pyruvic acid have been cleared up, there is, judging from a search of the recent literature (18), no agreement concerning the rate of fermentation of this important compound. Second, it was regarded as necessary to demonstrate that the yeast can be protected also against the aforementioned injurious effect of the unphysiological acid; *i.e.*, that the capacity of pyruvic acid to inactivate yeast can be decreased even during relatively long contact of the yeast with the acid.

It is believed that these view-points are met by presenting the following experiments.

In the experiment shown in Fig. 9, 10 cc. of a 20 per cent top yeast suspension were treated by bubbling ethylene through it for 20 minutes. 10 cc. of a 2 per cent pyruvic acid solution were then added, reducing in this way the yeast concentration to 10 per cent and that of the acid to 1 per cent. The bottom curves (*a*) show the fermentation of pyruvic acid, indicating that not only the permeability of the treated yeast was remarkably increased, but that in 26 hours about 95 per cent of the pyruvic acid had been fermented. The yeast was then washed three times with 70 to 80 cc. of tap water, separated by centrifuging, then suspended in carbon dioxide-saturated tap water, and used in the fermentation of 1 gm. of glucose (top curves (*b*)). The

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Curves a b c d
Control ○ ○ ○ ○
Treated ● ● ● ●
cm³ CO₂

significance of the protection obtained by the use of ethylene is evident.

In the experiment represented by Fig. 10, the conditions were somewhat altered in that 0.5 per cent pyruvic acid was fermented in a 5 per cent yeast suspension (curves (*a*)). The period of ethylene treatment was 10 minutes.

The pyruvic acid fermentation was followed, without washing the yeast, by three subsequent sugar fermentations, the course of which may be easily recognized by the steeper curves (*b*) and (*c*) (representing 0.5 gm. of glucose) and curves (*d*) (representing 1 gm. of glucose).

In order to ascertain the effect of a more unfavorable relation between yeast available for fermentation and acid to be fermented, 1 per cent pyruvic acid was fermented by a suspension containing 2.5 per cent yeast, which was previously treated with ethylene for 20 minutes. After the completion of the pyruvic acid fermentation (curves (*a*), Fig. 11), the yeast was carefully washed three times, each time with 70 to 80 cc. of tap water, again suspended in carbon dioxide-saturated water, and allowed to act upon 1 gm. of glucose (curves (*b*)). Compared with Fig. 9, the inspection of the curves conveys evidence to the effect of a smaller number of active cells during the course of the sugar fermentation, which is, moreover, more vigorous in the experiment carried out with protected enzymes.

The ability to resist the harmful effects of pyruvic acid and the possible high content of cozymase shown by the top yeast used are also evident by the inspection of the curves (*a*) and (*b*) of Fig. 12, where experiments with bottom yeast are recorded.

The experiments shown in Fig. 12 were carried out by using suspensions containing 10 per cent bottom yeast acting on 1 per cent pyruvic acid. The upper curves (*a*) indicate the course of

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FIG. 10. Curves (*a*) represent the fermentation of a 0.5 per cent pyruvic acid solution with a 5 per cent suspension of top yeast and with a similar suspension of yeast treated with ethylene, curves (*b*) a subsequent fermentation of 0.5 gm. of glucose added following the completion of the pyruvic acid fermentation, curves (*c*) a second 0.5 gm. of glucose following the experiment represented by curves (*b*), and curves (*d*) a final fermentation of 1 gm. of glucose added following the experiment represented by curves (*c*).

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the pyruvic acid fermentation and show that it yields not more than one-fourth to one-fifth of the theoretical amount of carbon

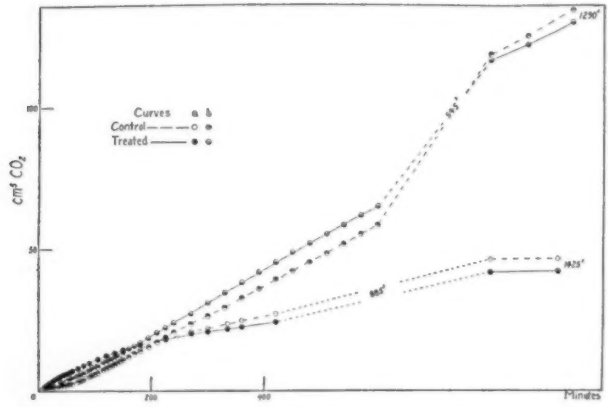

FIG. 11. Curves (*a*) represent the fermentation of a 1 per cent pyruvic acid solution with a 2.5 per cent suspension of top yeast and with a similar suspension of yeast which had been treated with ethylene, curves (*b*) the fermentation of 1 gm. of glucose with the washed yeast residues from the experiment represented by curves (*a*).

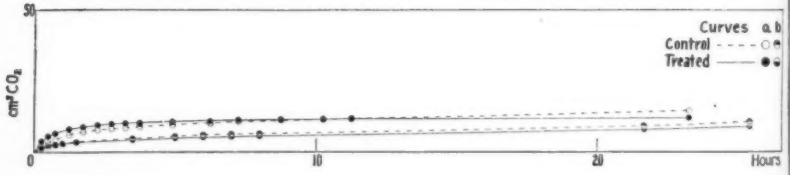

FIG. 12. Curves (*a*) represent the fermentation of a 1 per cent solution of pyruvic acid with a 10 per cent suspension of bottom yeast and with a similar suspension of bottom yeast treated with ethylene, curves (*b*) the fermentation of 1 gm. of glucose with the washed yeast residues from the experiment represented by curves (*a*).

dioxide. After a careful washing of the used yeast, its fermentability was tried out on glucose, but curves (*b*) indicate that the yeast was practically killed.

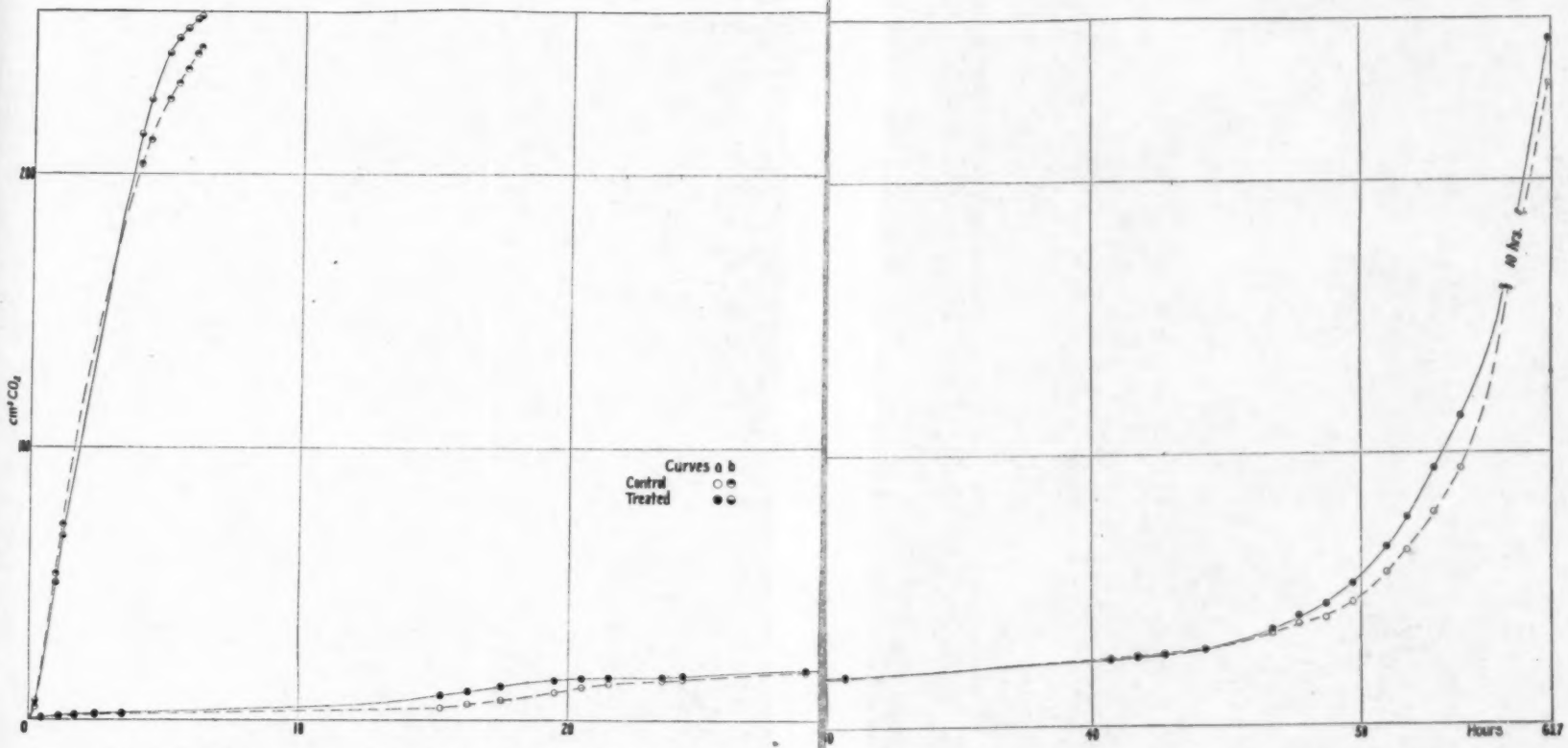

FIG. 13. The fermentation of glucose with a zymin preparation and with a zymin preparati treated with ethylene. Curves (*a*) the fermentation of an initial 1 gm. of glucose, and curves (*b*) the fermentation of a second 1 gm. of glucose by the same zymin preparation.

5. Experiments with Zymin.

Fig. 13 represents the course of an experiment carried out with zymin, the preparation of which is indicated on p. 32. The fermenting mash consisted of 1 gm. of zymin in 20 cc. of tap water, to which was added 1 gm. of glucose, after exposure to ethylene for 10 minutes. The inspection of the curves discloses a good agreement with previous experiments; however, the extraordinarily long induction time in the first pair of curves (*a*) is completely overshadowed by a fermentation rate, in the course of the fermentation of the subsequent gm. of glucose, only slightly lower than that produced by a mash made up with the same, but living yeast.

In view of the fact that Harden (19) regards zymin as "quite incapable of growth or reproduction, but produces a very considerable amount of alcoholic fermentation," this observation might be regarded to be in indirect agreement with the conclusion of Giaja (20), who regards it as not yet proved that the fermentation activity of living yeast cells has to be ascribed exclusively to the zymases present.

6. Fermentation of Calcium Hexose Diphosphate by Living Yeast.

Up to date it has been considered as proved that zymophosphates are unfermentable by living yeasts (21), and even recently this observation was thought to be confirmed.

The surprising observations on our top yeast suggested that we should again take up this question, especially since the unfermentability of these products by living yeasts has given rise, during the last few years, to numerous speculations. The I. G. Farbenindustrie A. G. in Elberfeld kindly put at our disposal a purified, insoluble calcium salt, which according to a communication of Dr. Hoerlein, contained only 13.64 per cent calcium and 11.08 per cent phosphorus, instead of 18.41 per cent calcium and 14.28 per cent phosphorus. From this salt the easily soluble magnesium salt was prepared, which is mentioned for the first time by Young (22). After the fermentation for 44 hours of a 2 gm. suspension of the calcium salt in 20 cc. of a 20 per cent top yeast suspension, there was obtained a quantity of carbon dioxide which was practically equivalent to the amount of sugar which,

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in accordance with the analysis, could be considered as uncombined.

The fermentation of the magnesium salt under the same conditions yielded, after 24 hours, practically no carbon dioxide.

CONCLUSIONS.

1. Zymase solutions were prepared which not only maintained their full activity for 65 days, but showed an increased activity.

2. Ethylene produces the effect of increasing the permeability of single cells and cells in tissues.

3. Enzymes outside of or bound to cells can be charged with an adsorbed film which may act as a protector against damaging transformation products.

This work has been aided as the result of a fellowship from the International Education Board to the senior author, who takes pleasure in acknowledging his indebtedness to Dr. R. A. Gortner and Dr. C. H. Bailey, of the Division of Agricultural Biochemistry, for the hospitality and consultations which he has enjoyed while at the University of Minnesota.

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IMPROVED PROCEDURE FOR THE EXTRACTION OF THE OVARIAN HORMONE.

II. SOME CORRECTIONS AND ADDITIONS.*

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During the 2 years following the publication of our improved procedure for the extraction of the ovarian hormone from liquor folliculi, many new and interesting features have been encountered. The purpose of the present paper is to make such corrections of the earlier description as are necessary and to add to the procedure the steps that we take to transfer the partially purified hormone to aqueous solution.

In order to attain greater clarity in recording the essential details of the modifications, the main points of the procedure of our earlier paper (Ralls, Jordan, and Doisy, 1926) are given. After distillation of the alcohol used for extraction of the liquor folliculi, the dry residue is dissolved in dilute alkali. This aqueous solution is then extracted with ethyl ether (Stage A), the ether distilled, and the residue transferred with 95 per cent alcohol to a separatory funnel. Enough water is added to reduce the concentration of alcohol to 70 per cent, and some of the contaminating substances including cholesterol are removed from the hormone by extraction with petroleum ether (Stage B). The alcoholic solution of the hormone is treated by a modified procedure which is described in detail in the paragraph headed Stage C.

Stage A.—Occasionally it has been found that peroxides have developed in the ether used for the extraction of the hormone from

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the aqueous solution of the soaps. That this may cause difficulty in the later steps of purification will be brought out in the discussion of our tables. Though the hormone is extremely sensitive to oxidizing agents, the peroxide in ethyl ether seems to cause little trouble with crude preparations and it is only after or during the purification with petroleum ether that the oxidation assumes any quantitative significance.

The modification of this part of the procedure then is: (1) the use of peroxide-free ether for the extraction of the hormone, and (2) the immediate removal of the ether by distillation as soon as the extraction is finished. The ether is distilled off and to make doubly sure of the absence of peroxides, some of the distillate is tested with aqueous KI for peroxide. Thus far we have found that if peroxide-free ether is used for the extraction and the ether extract distilled promptly after the completion of this step, the KI test is negative. After the distillation of the ether, a small volume of alcohol is added to the oily residue, the alcohol heated to boiling, cooled to $-10°$, and filtered. The residue is washed from the filter paper back to the original flask with alcohol and the leaching repeated. Three or four repetitions are sufficient to remove all of the hormone from the insoluble lipid material and a total volume (distributed through four or five leachings) of 100 to 150 cc. of alcohol is sufficient to remove the entire activity of the extract of 2 liters of liquor folliculi. The insoluble residue may be discarded as its activity is negligible. It has been our experience that this alcoholic solution is a good storage point; its activity seems to remain undiminished even after long periods of standing.

Stage B (Petroleum Ether Removal of Cholesterol, Etc.)—At this stage one-half or even more of the activity may be lost if the ethyl ether used in the earlier steps contained or was allowed to develop peroxides. In addition to this source of difficulty we have encountered still another. Several of our samples of petroleum ether liberate iodine from aqueous solutions of KI, which leads us to believe that they contain peroxides. It has therefore become our practice to test the petroleum ether as well as the ethyl ether with aqueous potassium iodide immediately before using them in the hormone work. Using tested solvents, we have no difficulties with the petroleum ether purification of the hormone.

If we may interpret the experiments of Glimm and Wadehn

(1926) in the light of our unfortunate experiences with peroxides in ethyl ether and petroleum ether, it seems rather probable that their unaccountable losses of activity were due to oxidation of a similar nature. Certain of our earlier experiments in which loss of potency occurred are being repeated owing to the suspicion that possibly the presence of peroxides in solvents has obscured the true behavior.

The removal of cholesterol with petroleum ether has proved to be very satisfactory in the hands of others, the principle of this separation having been adopted by Slotta (1927). We have altered the procedure only in the concentration of alcohol used. The alcoholic solution prepared in Stage A is diluted with an equal volume of water which makes the concentration of alcohol in the hormone solution about 47 per cent by volume. Five extractions are made with redistilled peroxide-free petroleum ether, one-fourth the volume of the aqueous alcoholic solution of hormone being used each time. As pointed out in our earlier paper the distribution ratio of the hormone is such that very little loss occurs but over 95 per cent of the contaminating substances are removed.

Stage C.—The dilute alcoholic solution of hormone is distilled and the residue leached with peroxide-free redistilled ethyl ether. The ethereal solution is immediately distilled and the residue remaining is taken up in redistilled 95 per cent alcohol.

In our earlier publication the opinion was expressed that the ethereal solution of this step would furnish a good stopping place for the accumulation of a large quantity of material for further chemical work. Our later work has definitely disillusioned us on this point, but mere distillation of the ether and the addition of alcohol solve the difficulty.

Stage D (Aqueous Solutions).—As a result of implantation experiments (Zondek and Aschheim, 1925) Zondek (1927) seems to have expressed the idea to Laqueur that the ovarian hormone should be obtainable in an aqueous solution. Two groups of investigators—the one headed by Zondek and the other by Laqueur—shortly after reported the preparation of aqueous solutions of the ovarian hormone (Folliculin B, Zondek and Brahn (1925); and Menformon, Laqueur, Hart, deJongh, and Wijsenbeek (1926) respectively). Other investigators, Glimm and Wadehn (1926), Dickens, Dodds, and Brinkworth (1927), Slotta (1927), and

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Biedl (1927), have subsequently reported the preparation of aqueous solutions.

Two different view-points of aqueous solubility must be considered: (1) the extraction of the hormone from ovaries or other tissue with water, and (2) the actual solubility of the refined product in water.

Laqueur (Laqueur, Hart, deJongh, and Wijsenbeek (1926) and Laqueur, Hart, and deJongh (1927)) has sought to avoid the preliminary extraction with alcohol and has to a certain extent succeeded. It is true that his procedures give active extracts but in our hands the losses in the residual tissue are too great to warrant serious consideration being given the process. This, however, may be due to our inability to follow the rather sketchy details of the description, or to Laqueur's willingness to sacrifice yield to secure speed and a high degree of purity of the final product.

Dickens, Dodds, and Brinkworth (1927) have attempted to shorten and improve the extraction by applying the picric acid process used with insulin. However, Laqueur (Laqueur, Hart, and deJongh, 1927) is unable to agree with their claim that their procedure leads to a good product which is water-soluble.

The true aqueous solubility can scarcely be doubted any longer. Both Zondek and Laqueur have adduced evidence that a rather refined product (Laqueur (1927), 0.001 to 0.002 mg. per 1 rat unit) forms a perfectly clear transparent aqueous solution, and the latter has found that the hormone diffuses through parchment and collodion. We have rather hesitated to adopt this view because of the known influence of one substance upon the solubility of another but since our recent success in reducing the rat unit to considerably below 0.001 mg. and thus eliminating much of the contaminating material, we are willing to subscribe to the possibility of aqueous solubility of the hormone. However, we cannot agree with Laqueur's statement that, as the hormone is purified, it loses its solubility in lipoid solvents. Certainly our best preparations are still soluble in ether and alcohol. We believe that the question of solubility is so connected with the nature of the contaminating substances that authoritative information must await the actual isolation of the chemical substance.

In view of the recently published data regarding potency (Laqueur, 0.001 to 0.002 mg. per 1 rat unit) solubility in water may be

an exceedingly confusing expression. The solubility in this case measured in physiological activity may be remarkably great, yet in the usual chemical terms exceedingly minute. For example, 10 rat units per 1 cc. of the product just referred to are 0.01 mg. per 1 cc. or 1 mg. per 100 cc., which is little greater than the solubility of many of the salts used in quantitative analytical chemistry.

Preparation of Aqueous Solutions.

After the final alcoholic solution of the hormone is obtained, it is merely diluted with an appropriate volume of water and the alcohol distilled off. Upon cooling, the solution becomes opalescent. A drop of dilute HCl is added to facilitate the flocking of the colloidal impurities and the solution kept in the refrigerator (1°) for a few hours. After filtration through a thick layer of asbestos, the filtrate should be indistinguishable in appearance from distilled water. The considerable portion of the active material which remains on the filter may be recovered by extraction with a small volume of alcohol followed by a repetition of the transference to aqueous solution as just described.

In summarizing the additions to our procedure, attention should again be called to the necessity of using solvents free from peroxides and to the importance of avoiding the storage of the hormone in solvents which may develop peroxides. The conversion of an alcoholic solution of the hormone into a crystal-clear aqueous solution is a simple matter.

DISCUSSION.

The stability of preparations of a physiologically active substance is of importance to the chemist engaged in its isolation and to the clinician who may employ it in the treatment of his patients. Some of our extracts have been kept for a sufficient period of time to warrant a brief discussion.

One of our best extracts was dissolved in ethyl alcohol in 1923. Subsequently, aliquots have been taken for assaying. Occasional tests have shown that the potency has not diminished during the $4\frac{1}{2}$ years of storage in the alcoholic solution.

A crude preparation dissolved in ethyl ether has been stored for about 2 years without any diminution of potency. This is a strik-

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ing contrast to the behavior of ethereal solutions of purified preparations which might lead one to suspect the presence of a protective complex in the crude extract.

Properly prepared aqueous solutions seem to retain their potency unimpaired for long periods of time. Our oldest aqueous preparation which was made in December, 1925, is still as active as it was at that time. Another extract made in this laboratory in June, 1926, has retained its full potency whereas an experimental commercial extract has lost about 35 per cent of its strength.

A petroleum ether solution prepared 3 years ago has lost four-fifths of its potency.

Turning our attention to the stability of the highly purified preparations, we may say that both aqueous and alcoholic solutions seem to retain their potency over long periods of time under proper conditions. The most essential condition for stability is the absence of oxidizing agents. Giesy (1920) has already called attention to the destruction of the hormone by a variety of oxidizing agents such as potassium permanganate and bromine. We can add an illustrative experiment in which an unsuspected source of oxidation caused us a great deal of trouble.

If a few cc. of redistilled ethyl ether are added to a tube and the ether allowed to evaporate, a scarcely visible droplet or two may remain. If a highly purified aqueous solution of the hormone is added, a few days suffice to effect a remarkable diminution of potency. In one experiment the residue from 3 cc. of ethyl ether destroyed 5 rat units, in another the residue from 6 cc. destroyed over 13 units. These values must not be regarded as the extent of destruction possible with the volumes of ether used but rather the point at which the experiment was terminated. In the second experiment a volume equivalent to 13 rat units of the original solution was insufficient to produce estrus in a spayed rat; a larger volume was not injected.

Semiquantitative experiments have shown that the residues or the higher boiling fractions of ethyl ether and petroleum ether contain most of the peroxides. Consequently it is conceivable that the use of volatile solvents containing peroxides for the extraction of the hormone may lead to a concentration of the peroxides in the residue containing the active material when the solvent

is distilled off, which may lead to serious losses of the hormone during subsequent purification.

The data of Table I are cited to show how the peroxides may interfere in the further steps of purification. The crude preparations stored as ethereal solutions seemed to possess about the activity expected from the volumes of liquor folliculi extracted. However, when an attempt was made to apply the petroleum ether-

TABLE I.

Effect of Organic Peroxides. Recovery of Activity after Petroleum Ether Extraction of Alcoholic Solution of Hormone.

Preparation No.	No. of petroleum ether extractions.	Rat units.		Recovery.	Remarks.
		Before petroleum ether extraction.	After petroleum ether extraction.		
				per cent	
20	6	870	650	74	
21	6	1260	630	50	
22	6	840	310	37	Conducted in refrigerator.
230	4	200	126	63	Used specially purified petroleum ether.
241 e	5	550	370	67	
25	5	500	330	66	
26	5	400	350	87	Same ethereal solution but distribution of No. 126 carried out 1 year later than No. 26.
126	5	575	115	20	
30	4	32	30	94	
301 a	5	1295	720	56	Distribution of No. 30 carried out after ether solution had been kept 5 mos.
Average recovery with Nos. 26 and 30 omitted.....				54	

70 per cent alcohol distribution for purification, unaccountable losses of potency occurred. In working up the details of the process originally, we were pleased to find that a very advantageous increase of purity could be attained with practically no loss of activity. Upon further application of the procedure losses of such a magnitude were encountered that the method was of no service. After some casting about for a possible cause, our suspicion fell

upon the solvents, some of which upon examination were found to contain peroxides. The explanation now seems to be fairly clear. For instance, Preparation 26 (Table I) retained its potency in ethereal solution unimpaired for 1 year. When it was still fresh and presumably no peroxide was present, it was purified with a recovery of 87 per cent of the rat units. 1 year later a similar number of rat units (Preparation 126) was purified by the same process but in this case only 20 per cent of the active material was

TABLE II.

Effect of Antioxidants. Recovery of Activity after Petroleum Ether Extraction of Alcoholic Solution of Hormone.

Preparation No.	No. of petroleum ether extractions.	Rat units.		Remarks.
		Before petroleum ether extraction.	Recovered after petroleum ether extraction.	
25 e	4	150	120	Small amount cod liver oil, soap, and Na_2SO_3.
26 c	4	88	88	5 mg. catechol.
26 f	5	44	44	Few mg. hydroquinone.
30 b	8	45	37	Few mg. catechol added with each volume of petroleum ether.
30 c	8	45	37	Few mg. formic acid added with each volume of petroleum ether.
30 d	8	45	37	Few mg. $NaHSO_3$ added with each volume of petroleum ether.
30 f	8	45	32	Few mg. hydroquinone added with each volume of petroleum ether.
37	5	1280	1200	Shook ether solution with alkaline glucose.

recovered and even this amount decreased rapidly during storage of the purified preparation. The pair, Preparations 30 and 301 a, bring out the same point but in a less striking degree.

Casting about for some method of saving the hormone of our stock solutions which contained peroxide, we tried the use of antioxidants (Moureu and Dufraisse, 1927) without striking success. Sometimes (Table II) an antioxidant appeared to be effective but subsequent trial gave a less favorable result. The effect of a strong reducing agent was also tried with Preparation 37. The

ethereal solution which gave a strong test for peroxides was shaken with 2 portions of an alkaline (NaOH) solution of glucose. Upon subsequently testing for peroxides, their presence could not be detected. The recovery of 94 per cent of the potency of this preparation after purification is regarded as satisfactory.

In Table III we present data upon the recovery of potency when due care was taken to exclude peroxides from the solvents used in the purification. Such a procedure is feasible and seems to be

TABLE III.

Peroxides Excluded. Recovery of Activity after Petroleum Ether Extraction of Alcoholic Solution (50 Per Cent) of Pituitrin.

Preparation No.	No. of petroleum ether extractions.	Rat units.		Recovery.	Remarks.
		Before petroleum ether extraction.	After petroleum ether extraction.		
				per cent	
31	5	400	355	89	Diminished illumination.
32	5	686	528	77	" "
33	5	480	480	100	" "
35		2000	1666	83	
341 f	5	1690	1500	89	Ordinary daylight.
36	5	475	475	100	" "
361 b	5	2460	2460	100	" "
37	5	1280	1200	94	" "
38	5	1000	1200	120*	" "
381 c	5	1000	1200	120*	" "
39	5	2080	2000	96	" "
Average.....				92	

* Omitted from the average.

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entirely satisfactory. The average recovery obtained in Table I is 54 per cent as contrasted with the recovery of 92 per cent in Table III. A word might be said about the omission of Preparations 38 and 381 c from the calculation of the average. A recovery of 120 per cent of the potency is scarcely to be expected but, when one considers the uncertainty of the biological assay, it is not surprising. Some of our lower values, as 77 per cent in Preparation 32, may likewise be due to errors of testing. If we should

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admit this and trust that the plus and minus errors compensate, then our average recovery becomes 97 per cent.

In the light of our experimental misfortunes due to the presence of peroxides in solvents, we want to reiterate the warnings of Rosenheim (1927) and Holm (1928). Though their papers dealt with the possibility of destroying vitamins by oxidation with peroxides of the solvents, the warning should be extended to include all biological substances of such extreme activity that they

TABLE IV.

Preparation of Aqueous Solution of the Ovarian Hormone.

Preparation No.	Rat units in alcohol solution.	Rat units in aqueous solution.	Rat units in aqueous solution.	Rat units per 1 cc.	Remarks.
			per cent		
31 y	150	132	88	2	Water-clear.
312 e	1240	1025	83	25	" Two aqueous extractions.
331 c	480	302	63	7	Water-clear.
34 f	1500	704	47	22	Slight opalescence.
35	1666	1320	79	16	Water-clear.
36 c	475	394	83	12	" Two aqueous extractions.
361 c	2460	1475	60	24	
360 c	1680	1450	86	42	1 rat unit = 0.0033 mg.*
37 c	960	480	50	10	Water-clear. Two aqueous extractions.
38 c	1200	940	78	27	Opalescent.
381 c	1200	937	78	14	
382 d	1330	1132	85	33	1 rat unit = 0.0029 mg.
43	2500	1640	65	44	Combined residues.

* Subsequently purified to over 1000 rat units per 1 mg.

are measured in physiological rather than metric units. The loss of potency of 1000 rat units is actually the oxidation of less than 1 mg. of the hormone.

Preparation of Aqueous Solutions.

Our early doubts about the aqueous solubility of the hormone were really based upon two points: (1) the incompleteness of the extraction from tissues without the use of organic solvents; (2)

the presence of a faint turbidity in aqueous solutions of our purest preparations. The first point is still of importance but the second is no longer of consequence. Adequate filtration will remove the turbidity leaving a clear, colorless, sparkling solution which possesses potency.

By the principles outlined in an earlier section of this paper, we have prepared a large number of clear aqueous solutions. In no instance, however, have we obtained the full number of rat units in the filtrate, the residue on the filter always containing some active material. Preparation 43, Table IV, represents a solution prepared from the combined insoluble residues of earlier preparations. By working over the precipitates practically all of the potent material may be transferred to a water-soluble form.

We have altered the procedure for the preparation of aqueous solution so frequently that a definite set of conditions cannot be described. It has seemed that the actual recovery of rat units in the aqueous solution is highest when the hormone in a solvent miscible with water is added to water, and the organic solvent distilled off. The quantity of water used has varied. For example, the volume of the filtrate in Preparation 360c was 35 cc. and in Preparation 37c, 48 cc.; the recovery in the first case was 86 per cent but only 50 per cent in the latter.

SUMMARY.

1. Attention is called to the possibility of the destruction of the ovarian hormone by oxidation by the peroxides present in ethyl ether and petroleum ether.

2. The preparation of aqueous solutions of the ovarian hormone is described.

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STUDIES ON BLOOD CELL METABOLISM.

II. THE EFFECT OF METHYLENE BLUE AND OTHER DYES UPON THE GLYCOLYSIS AND LACTIC ACID FORMATION OF MAMMALIAN AND AVIAN ERYTHROCYTES.

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In the preceding paper (1) we have shown that the oxygen consumption of mammalian erythrocytes is enormously accelerated in the presence of minute amounts of methylene blue and certain other dyes. This effect upon the respiration of the mammalian cells differs from the effect of methylene blue upon the erythrocytes of avian blood. In the present paper we present the results of a continuation of this study; namely, a comparison of the influence of methylene blue upon glycolysis and lactic acid formation in mammalian erythrocytes, and in the actively respiring cells of avian blood.

The extensive literature upon glycolysis in the blood has been reviewed by other observers. Lepine (2) first introduced the term and showed that the glycolytic process in blood required the presence of a ferment. Since glycolysis did not occur in serum he stated that it was in some way connected with the blood cells and suggested that the ferment was located in the leucocytes. The important work of Levene and Meyer (3) demonstrated the rôle of leucocytes in glycolysis. Rona and Arnheim (4), as well as others, have shown that glycolysis is effected by erythrocytes as well.

The studies of recent years have indicated that the disappearance of glucose from the blood (and other tissues) occurs in two different ways. In the first, an actual oxidation with considerable liberation of energy occurs; the second is effected by a splitting of the hexose molecule into 2 molecules of lactic acid, but without a

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coincident utilization of oxygen. The latter process has rather striking analogy to the fermentation of yeast cells. It is possible, indeed probable, that some of the sugar first converted into lactic acid is further broken up by oxidation, in which case it falls in the first category.

In order to determine the relative amounts of glucose which disappear by these two paths, a knowledge of the amount of lactic acid produced is required as well as of the amount of glucose which is lost. Since 2 mols of lactic acid are formed from the glycolysis of each mol of sugar, the glucose which is split up in excess of this simple proportion must then disappear by the other route. It is convenient to express this relation by means of what we may call the *glycolytic quotient*, as follows:

$$\frac{\text{Millimols lactic acid produced}}{2 \times \text{millimols glucose degraded}}$$

When this quotient is unity, it indicates that all of the glucose has been converted into lactic acid. When the ratio is less than unity, the fraction expresses the proportion of the total glucose converted to lactic acid, the remainder having disappeared by another channel or by further splitting of lactic acid. A study of this *glycolytic quotient* as found in adult mammalian erythrocytes in blood prepared and freed from leucocytes by the methods detailed in Paper I (1), and incubated as previously described, demonstrated the fact (Tables I and II) first predicted by Claude Bernard[1] that the sugar loss can usually be accounted for in very

[1] It is of interest that the relation between the disappearance of sugar and the formation of lactic acid was first postulated by Claude Bernard who considered that it was a fermentation.

"Ce ferment lactique se rencontre dans le sang, dans les muscles, dans le foie lui-même; car, j'ai constaté que les muscles et divers tissus ne deviennent acides après la mort qu'autant qu'ils renferment du sucre ou de la matière glycogène qui subit très-rapidement une fermentation lactique. J'ai reconnu autrefois [Cours de physiologie, au Collège de France, t. I, pp. 379–392, 1855] cette fermentation lactique de la matière glycogène, d'abord dans les muscles du fœtus, où elle présente son *summum* d'intensité; je l'ai constatée plus tard chez l'homme et les animaux adultes. J'ai vu également la fermentation lactique dans le sang. Quand on prend sur un animal qu'on vient de sacrifier le sang le plus sucré, celui des veines sus-hépatiques, on constate que sous l'influence d'une chaleur modérée

large part by new formation of lactic acid. This glycolysis, which requires no oxygen, is in harmony with the observation that adult mammalian erythrocytes have a scarcely measurable oxygen consumption. The carbohydrate metabolism of the mammalian erythrocyte thus appears to occur without oxygen under normal conditions. In contrast to the *glycolytic quotient* of mammalian blood, that of avian blood is always less than 1, indicating that some oxidative process plays a share in the fate of the sugar which disappears, a fact in agreement with the active oxygen consumption found in nucleated red blood cells.

Action of Methylene Blue and Similar Dyes upon the Glycolysis and Lactic Acid Production of Erythrocytes.

The investigations of Loeb (5) and of Melvin (6) showed that ox and sheep blood under aerobic conditions have very slight glycolysis *in vitro*. A similar behavior on the part of avian blood was later shown by Rüter (7). Glycolysis is more marked in the blood of the dog and of man.

(*a*) *Mammalian Blood.*—The addition of methylene blue[2] produces a lowering of the *glycolytic quotient* in mammalian blood. It increases the amount of sugar destruction, but its chief effect

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le sucre disparaît du sang en lui donnant une réaction acide, au lieu de la réaction alcaline normale. . . . . J'admets donc, quant à moi, que la destruction du sucre a lieu par *fermentation* et non par l'influence directe des alcalis du sang, qui favorisent seulement cette réaction. . . . . Si l'on prend deux parties égales du même sang sucré et si l'on fait cuire l'une d'elles de façon à coaguler ou à détruire le ferment lactique, on voit le sang rester alcalin et le sucre persister pendant très-longtemps dans la liqueur." (Bernard, C., Leçons sur le diabète, Paris, 1877, 328.)

[2] The concentration of methylene blue and of the other dyes used, as in the previous communication, was 0.005 per cent unless otherwise indicated. The same specimens of dyes were employed. Blood sugar determinations were made by Benedict's modification of the Folin-Wu technique. The lactic acid was determined by the modification of Clausen's method introduced by Friedemann, Cotonio, and Shaffer. For each experiment 10 cc. of blood were used. Triplicate blood sugar estimations and duplicate lactic acid estimations were made. The limits of error of the analytic procedures are estimated to be per liter 0.2 mM for lactic acid and 0.25 mM for sugar, under the conditions of these experiments.

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is to lessen the coincident lactic acid production (Table I).[3] We interpret the meaning of these results to be that methylene blue causes a shift of the course of carbohydrate catabolism away from the (anaerobic) formation of lactic acid and toward the oxidative

TABLE I.

Mammalian Erythrocytes. Comparative Study of Glycolysis and Lactic Acid Formation as Influenced by the Oxidative Action of Methylene Blue (M.B.) (0.005 Per Cent Concentration).

Experiment No.		Blood glucose, mM per l.			Lactic acid, mM per l.			Calculated glycolysis in blood with M. B.	Oxidation by M. B., calculated as glucose.	Glycolytic quotient.
		Before incubation.	After 3 hrs. incubation.	Decrease.	Before incubation.	After 3 hrs. incubation.	Increase.			
								mM per l.	*mM per l.*	
1	Dog blood.									
	Control	3.96	2.19	1.77	2.19	5.73	3.54			1.00
	Added M.B.		2.01	1.95		4.47	2.28	1.14	0.81	0.58
2	Control	4.50	2.00	2.50	3.87	8.66	4.79			0.96
	Added M.B.		1.61	2.89		6.00	2.13	1.12	1.77	0.37
3*	Human blood.									
	Control	4.28	2.03	2.25	1.92	6.03	4.11			0.91
	Added M.B.		0.94	3.34		5.00	3.08	1.69	1.65	0.46
4*	Control	4.22	2.12	2.10	2.73	6.90	4.17			0.99
	Added M.B.		1.38	2.84		6.31	3.58	1.80	1.04	0.63
5*	Control	5.98	3.41	2.57	2.71	7.81	5.10			0.99
	Added M.B.		2.02	3.96		7.77	5.06	2.55	1.41	0.64

* The blood used in Experiment 3 was from a patient with heart disease, in Experiment 4 from a patient with polycythemia, and in Experiment 5 from a patient with hyperthyroidism.

path. The action of methylene blue is most striking in the experiments upon blood with low glycolytic power (Table II). In these

[3] For example, in Table I, Experiment 1, the *glycolytic quotient* reached unity in the control sample, indicating that no oxidation had taken place. In the sample containing methylene blue, however, the *glycolytic quotient* was reduced to 0.58. In this case the lactic acid increase was 2.28 millimols, an amount corresponding to a sugar decrease of 1.14 millimols. As the glucose decrease found however was 1.95 millimols, we may conclude

experiments the amount of sugar which disappears in the presence of methylene blue is quite as large as it is in control specimens, which contain no dye, but there is practically no new formation of lactic acid.[4]

(*b*) *Avian Blood.*—The *glycolytic quotient* in the case of avian blood is in general lower than in the case of mammalian blood, a fact in accord with its higher respiratory metabolism. The experiments on chicken blood, however, examined twice from different birds showed neither disappearance of glucose nor methylene blue action (Table II). It is stated by Bornstein and Ascher (8) that although normal goose blood shows no glycolysis, anemic goose blood has some glycolytic power. In our experiments, no glycolysis was found in blood taken from one goose until after the bird had been bled repeatedly and thus rendered anemic. Material from a second normal goose, however, showed glycolysis even in the first sample taken. The effect of methylene blue upon the *glycolytic quotient* in goose blood is similar to that in mammalian blood.

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that 1.14 millimols disappeared by glycolysis and the remainder (0.81 millimols) by oxidation.

Where the *glycolytic quotient* is less than 1 in the control, the corresponding amount of sugar has disappeared by a path other than that of lactic acid formation. The amount of glucose which is consumed coincident with the new lactic acid formation in the methylene blue experiment is then determined on the basis of that produced in the control. This amount deducted from the total amount of glucose which disappears in the methylene blue experiment gives the amount oxidized by reason of the presence of the methylene blue itself. For example, in Table II, Experiment 3, the *glycolytic quotient* is 0.59, indicating that a very large proportion (41 per cent) of the glucose has disappeared by the oxidative path. This must be taken into account in estimating the methylene blue effect, as follows: Since 1.01 millimols of lactic acid were produced in the control during the disappearance of 0.86 millimols of sugar, the production of the 0.27 millimols of lactic acid found in the specimen to which methylene blue was added was associated on this basis with the loss of 0.23 millimols of sugar. The difference between this amount and the total glucose which disappeared (0.89 millimols − 0.23 millimols = 0.66 millimols) is that oxidized by the methylene blue.

[4] For example, in Experiment 1, Table II, the *glycolytic quotient* of the control blood is equal to unity, while in the methylene blue sample the quotient has fallen to 0.01. This fall is due to the large amount of sugar (0.73 millimols) which has disappeared by oxidation and without coincident increase of lactic acid.

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(c) *Effect of Other Dyes.*—In accordance with our findings in the preceding paper on the respiratory metabolism of erythrocytes, the oxidative action of methylene blue upon glucose in the blood is shared by other dyes which Clark (9) has shown to possess similar

TABLE II.

Mammalian and Avian Blood with Low Glycolytic Power. Comparative Study of Glycolysis and Lactic Acid Formation. Oxidative Action of Methylene Blue (M.B.) (0.005 Per Cent Concentration).

Experiment No.		3 hrs. incubation.						Calculated glycolysis in blood with M.B.	Oxidation by M.B., calculated as glucose.	Glycolytic quotient.
		Blood glucose, mM per l.			Lactic acid, mM per l.					
		Before incubation.	After incubation.	Decrease.	Before incubation.	After incubation.	Increase.			
								mM per l.	*mM per l.*	
1	Ox blood.									
	Control.....	3.61	2.94	0.67	7.50	8.85	1.35			1.00
	M.B.....		2.78	0.83		7.52	0.02	0.10	0.73	0.01
2	Sheep blood.									
	Control.....	2.34	1.60	0.74	4.16	5.58	1.42			0.96
	M.B.....		1.52	0.82		4.21	0.05	0.03	0.79	0.03
3	Goose blood.									
	Control.....	6.39	5.53	0.86	6.18	7.19	1.01			0.59
	M.B.....		5.50	0.89		6.45	0.27	0.23	0.66	0.17
4	Chicken blood.									
	Control.....	14.72	14.68	0.00	3.25	3.27	0.00			No glycolysis.
	M.B.....		14.72	0.00		3.26	0.00		No oxidation by M.B.	

oxidation-reduction potentials. Studies have been made of the effect of toluylene blue, phenol indophenol, and Bindschedler's green (double zinc salt) in equimolar concentration. In each case there is an oxidizing action on the carbohydrates, as the *glycolytic quotient* and calculated glycolysis demonstrate (Table III). The

one exception is phenol indophenol, which in several experiments had little or no demonstrable action (Table III, Experiment 2). The calculated amounts of sugar per liter which have undergone oxidation in the presence of the various dyes, are indicated.

TABLE III.

Mammalian Blood. Comparative Action of Methylene Blue (M B.), Phenol Indophenol (P.I.), Toluylene Blue (T.B.), and Bindschedler's Green (B.G.) upon Glycolysis and Lactic Acid Formation.

Experiment No.		Blood sugar, mM per l.			Lactic acid, mM per l.			Calculated glycolysis in blood with dyes.	Oxidation by dyes, calculated as glucose.	Glycolytic quotient.
		Before incubation.	After incubation.	Decrease.	Before incubation.	After incubation.	Increase.			
								mM per l.	*mM per l.*	
1	Human blood.									
	Control.....	4.28	2.03	2.25	1.92	6.03	4.11			0.91
	M.B.....		0.93	3.35		5.00	3.08	1.68	1.67	0.46
	P.I.....		1.53	2.75		6.36	4.44	2.43	0.32	0.81
2	Control.....	4.22	2.12	2.10	2.73	6.90	4.17			0.99
	M.B.....		1.38	2.84		6.31	3.58	1.80	1.04	0.63
	P.I.....		2.18	2.04		6.80	4.07	2.05	0.00	1.00
3	Control.....	5.97	3.40	2.57	2.71	8.00	5.29			1.00
	M.B.....		2.02	3.95		7.96	5.25	2.55	1.40	0.66
	T.B.....		1.90	4.07		7.13	4.42	2.14	1.93	0.54
4	Control.....	5.05	2.65	2.40	2.13	6.26	4.13			0.86
	M.B.....		1.54	3.51		5.84	3.71	2.15	1.36	0.53
	B.G.....		2.13	2.92		5.69	3.56	2.07	0.85	0.61
5	Dog blood.									
	Control.....	4.50	2.00	2.50	3.87	8.66	4.79			0.96
	M.B.....		1.61	2.89		6.00	2.13	1.11	1.78	0.37
	T.B.....		1.56	2.94		7.20	3.33	1.74	1.20	0.57

Effect of Concentration of the Dye.

In harmony with its effect upon the respiratory metabolism, methylene blue exerts its action upon glycolysis and lactic acid formation over a considerable range of concentration. No close relationship was observed between the amount of methylene blue added and the magnitude of the effect on the *glycolytic quotient* or

on the amount of glucose oxidized (Table IV). Methylene blue is active at a concentration as low as 0.0005 per cent. At 0.0001 per cent the oxidizing action is greatly reduced, while it ceases at 0.00005 per cent. The limits of the range of effective concentration of the methylene blue are approximately those found for its effect upon respiration. The wide range of action suggests that the mechanism is one of catalysis. Moreover since methylene blue-methylene white is a highly reversible oxidation-reduction

TABLE IV.

Blood Glycolysis and Lactic Acid Formation. Variation in Concentration of Methylene Blue (M.B.) in Dog Blood.

	Blood glucose, mM per l.			Lactic acid, mM per l.			Calculated glycolysis in blood with M.B.	Oxidation by M.B. calculated as glucose.	Glycolytic quotient.
	Before incubation.	After incubation.	Decrease.	Before incubation.	After incubation.	Increase.			
							mM per l.	*mM per l.*	
Control, no M.B.....	4.28	1.97	2.31	2.91	7.52	4.61			1.00
0.1 per cent M.B.....		2.31	1.97		4.82	1.91	0.97	1.00	0.48
0.05 " " "		2.14	2.14		5.20	2.29	1.10	1.14	0.53
0.01 " " "		1.98	2.30		5.00	2.09	1.05	1.25	0.45
0.005 " " "		2.04	2.24		6.00	3.09	1.55	0.69	0.69
0.001 " " "		2.00	2.28		6.00	3.09	1.55	0.73	0.68
0.0005 " " "		1.94	2.34		5.55	2.64	1.32	1.02	0.57
0.0001 " " "		2.00	2.28		6.81	3.90	1.74	0.54	0.86
0.00005 " " "		2.05	2.23		7.35	4.45	2.22	No oxidation.	1.00

system, it is comprehensible that the concentration of the dye may have little influence on the oxidative process.

Velocity of Glycolysis and Lactic Acid Formation with Methylene Blue.

The rate of glycolysis appears to differ in the various animal species. Lepine (10) as the result of his experiments on dog blood found the curve of glycolysis to be parabolic in form, while Irving

(11) working with rabbit blood maintains that the rate of glycolysis is linear "over a wide range of values, the rate being about 18 mg. per cent per hour at 38°C." The initial concentrations of glucose and of lactic acid present undoubtedly may have influence upon the velocity of the process.

In some experiments in which the rate of glycolysis only was followed in human blood, the curve was more or less linear in type, while in experiments with dog blood the curve described by

TABLE V.

Rate of Glycolysis and Lactic Acid Formation in Dog Blood with and without Methylene Blue (M.B.), during the First 3 Hours of Incubation (37°).

	Blood sugar, mM per l.			Lactic acid, mM per l.			Glycolytic quotient.
	Before incubation.	After incubation.	Decrease.	Before incubation.	After incubation.	Increase.	
1 hr. incubation, control	4.28	3.08	1.20	2.91	5.13	2.22	0.93
1 " " M.B		2.70	1.58		4.14	1.23	0.39
After 2 hrs. incubation.							
Control		2.43	1.85		6.42	3.51	0.95
Added M.B		2.20	2.08		5.46	2.55	0.61
After 3 hrs. incubation.							
Control		1.97	2.31		7.56	4.65	1.00
Added M.B		1.62	2.66		5.78	2.87	0.53

Lepine was found. Table V presents the data from an experiment with dog blood while Fig. 1 represents the glycolytic curve in the control sample and in the sample containing methylene blue. The curves are similar, the chief difference being that methylene blue has accelerated the process. The lactic acid production in the control blood increases in proportion to the glycolysis as may be seen from the *glycolytic quotients*. In the sample containing methylene blue the *glycolytic quotient* is reduced.

Effect of Temperature.

The velocity of blood glycolysis, being governed by the general laws of enzyme action, is accelerated as the temperature increases until a certain optimum is reached. The action of methylene blue follows the same rule (Table VI). If the blood is subjected to a temperature of 56° for 1 hour in order to destroy the glycolytic ferment, the action of methylene blue is also stopped.

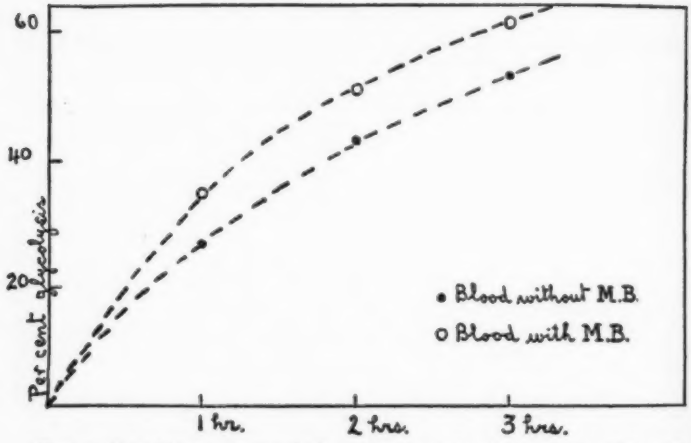

FIG. 1. Dog blood. Curve indicating the rate of glycolysis with and without methylene blue (0.05 per cent).

Action of Cyanides upon Glycolysis and Methylene Blue Oxidation.

According to Warburg's (12) theory, the activation of molecular oxygen by iron is the essential factor in biological oxidations. An important proof is furnished by the effect of cyanides. Warburg (13), from studies on the inhibiting effect of cyanides upon oxidation, asserts that the action is due to a removal of the metal catalyst (iron) to form complex inactive ions (ferro- or ferricyanides). The addition of cyanides on the basis of this theory should not necessarily affect the glycolysis of blood as this seems to require no oxygen. At the concentration of cyanides which we have used (0.001 to 0.002 N, dissolved in 0.9 per cent NaCl) this appears to

be the case. It was deemed important to determine whether the addition of cyanides would prevent the oxidizing action of methylene blue. Potassium cyanide at the concentrations stated above was added to blood and the samples were incubated as usual for 3 hours (38°). The oxidizing action of methylene blue was not at all impaired by the addition of cyanides, but proceeded at the same velocity as in the blood containing methylene blue but no cyanide (Table VII). This oxidation, then, is effected by methyl-

TABLE VI.

Effect of Temperature upon Blood Glycolysis with and without Addition of Methylene Blue (M.B.) (0.005 Per Cent). Defibrinated Dog Blood.

Duration of experiment, 150 minutes.

Experiment No.		Blood glucose.		Glycolysis.
		Before incubation.	After exposure to indicated temperature.	
		gm. per cent	*gm. per cent*	*per cent*
1	Kept at ice box temperature (1°).			
	Control	0.208	0.207	0
	Added M.B		0.208	0
2	At room temperature (22°).			
	Control		0.189	9.1
	Added M.B		0.173	16.8
3	At 37° (incubator).			
	Control		0.176	15.5
	Added M.B		0.145	30.3
4	Kept 1 hr. at 56°, then placed in incubator (37°).			
	Control		0.206	0
	Added M.B		0.206	0

ene blue in atmospheric air in the presence of cyanides and hence presumably without the catalytic action of iron. Thunberg (14) found that the addition of cyanide had no effect on the oxidation of succinic acid to fumaric acid in the presence of methylene blue in muscle preparations under strict anaerobiosis. The oxidizing action of methylene blue was inhibited by cyanide, however, when the experiment was carried on in atmospheric air. Warburg considered that this experiment corroborated his theory in that the

succinic acid and washed muscle in the presence of cyanide form a system which does not take up oxygen. In the glycolysis of blood, however, the oxidation of some intermediary carbohydrate seems to occur with methylene blue in the presence of air without the aid of the iron catalyst. The fact that the oxidizing action of methylene blue upon glucose is not disturbed by the presence of cyanides is in accordance with the lack of influence of cyanides upon methylene blue oxidation noted in the preceding paper.

TABLE VII.

Action of Potassium Cyanide upon Blood Glycolysis and Lactic Acid Formation, with and without Methylene Blue (M.B.) (0.005 Per Cent). Defibrinated Human Blood. Incubation at 37° for 3 Hours.

	Blood glucose, mM per l.			Lactic acid, mM per l.			Calculated glycolysis in blood with M.B.	Oxidation by M.B., calculated as glucose.	Glycolytic quotient.
	Before incubation.	After incubation.	Decrease.	Before incubation.	After incubation.	Increase.			
							mM per l.	*mM per l.*	
Control	3.38	1.33	2.05	3.21	6.15	2.94			0.72
Added M.B.		1.27	2.11		4.92	1.71	1.19	0.92	0.40
" 0.001 N KCN		1.39	1.99		7.11	3.90			0.98
" 0.001 " " + M.B.		1.39	1.99		4.89	1.61	0.82	1.17	0.40
Added 0.002 N KCN		1.39	1.99		6.33	3.12			0.78
" 0.002 " " + M.B.		1.36	2.02		4.81	1.60	0.81	1.21	0.40

Action of Phosphates.

The effect of methylene blue upon blood glycolysis in the presence of added phosphate was studied. Two kinds of experiments were performed. In one Sørensen's phosphate mixture (pH 7.4) and glucose (*d*-glucose Pfanstiehl) were added to blood prepared in the usual way, and the glycolysis and lactic acid formation were determined after 3 hours incubation. In the other, phosphates alone were added and the sugar decrease and lactic acid formation were compared in the samples with and without phosphates and

with and without methylene blue (Table VIII). The addition of phosphates appears to increase the oxidative effect of the dyes upon blood glucose.

TABLE VIII.

Action of Phosphates. Blood Glycolysis and Lactic Acid Formation with and without the Addition of Methylene Blue. Defibrinated Dog Blood.

Experiment No.		Blood glucose, mM per l.			Lactic acid, mM per l.			Calculated glycolysis in blood with M.B.	Oxidation by M.B., calculated as glucose.	Glycolytic quotient.
		Before incubation.	After incubation.	Decrease.	Before incubation.	After incubation.	Increase.			
								mM per l.	*mM per l.*	
1	Phosphate mixture (pH 7.4) with glucose added.									
	Control	12.97	9.54	3.43	2.97	7.06	4.09			0.60
	Added M.B		8.72	4.25		4.91	1.94	1.62	2.63	0.23
	" P.I		8.13	4.84		6.08	3.11	2.60	2.24	0.32
2	Phosphates and glucose added.									
	Control	16.33	13.54	2.79	5.92	10.64	4.72			0.81
	Added T.B		11.81	4.52		8.51	2.59	1.53	2.99	0.29
3	No phosphate added.									
	Control	3.38	1.33	2.05	3.21	6.15	2.94			0.72
	Added M.B		1.27	2.11		4.92	1.71	1.19	0.92	0.40
4	Phosphate mixture (pH 7.4) added.									
	Control		1.22	2.16		6.49	3.28			0.76
	Added M.B		1.27	2.11		4.39	1.18	0.78	1.33	0.28

M.B. = methylene blue.
P.I. = phenol indophenol.
T.B. = toluylene blue.

Integrity of Red Blood Cells in Relation to Glycolysis and Methylene Blue Action.

Many authors have observed that the glycolytic process seems closely related to the integrity of the cell surface. Loeb (5) suggested that glycolysis is an intracorpuscular process. Macleod

(15) repeated the suggestion, which correlates his observation that glycolysis is diminished in blood cells washed with saline solution, with the observations of Rona and Döblin (16) that washing rendered the corpuscles incapable of absorbing sugar. We have repeated Macleod's (15) experiments in order to compare glycolysis under such conditions with the oxidative action of methylene blue. The red cells were washed with Locke's mammalian solution instead of saline. It was found that washing of the blood cells decidedly impairs both normal glycolysis and methylene blue action (Table IX).

TABLE IX.

Effect of Alteration or Destruction of Erythrocyte Surface upon Glycolysis with and without Methylene Blue (M.B.). (Washing of Red Blood Cells and Hemolysis.)

Experiment No.		Blood glucose, mM per l.		Glycolysis.
		Before incubation.	After incubation (3 hrs.).	
				per cent
1	Control blood	6.39	5.00	21.7
	Added M.B.		4.36	31.7
	Blood washed twice with Locke's solution	11.00	10.10	8.2
	Added M.B.		9.45	14.0
2	Control blood	5.55	2.00	64.0
	Added M.B.		1.55	72.0
	Washed blood cells	5.05	3.33	34.0
	Added M.B.		2.27	55.0
	Hemolysis.			
3	Ox blood hemolyzed with water, control	10.00	10.00	0
	Added M.B.		9.94	0

Rona and Arnheim (4) in 1913 showed that hemolysis with distilled water stops glycolysis. These authors hemolyzed the blood with large amounts of water (20 cc. of blood were mixed with 180 cc. of water). We have compared the glycolysis in normal human blood with that of blood hemolyzed in two ways: (*a*) repeated freezing and thawing, and (*b*) hemolysis with distilled water. In the latter case the serum was removed by strong centrifugation and then replaced by an equivalent amount of water.

Glycolysis was impaired in the hemolyzed samples, but not stopped, as the following example shows:

Human blood.	Glycolysis. *per cent*
(*a*) Glycolysis after 3 hrs., 37°. Control	52
(*b*) Hemolysis by freezing and thawing	36
(*c*) Hemolysis with distilled water	18

In order to observe the action of methylene blue in a sample of blood in which glycolysis was entirely lacking, ox blood was employed. Here (Table IX, Experiment 3) hemolysis with distilled water stopped glycolysis, and methylene blue as well had no action.

TABLE X.

Effect of Hemolysis by Freezing and Thawing upon Defibrinated Goose Blood.

		Glucose, mM per l.		Glycolysis.
		Before incubation.	After incubation.	
				per cent
Sample A, clear supernatant fluid.	Control.	5.25	5.22	0
	Added methylene blue.		5.25	0
Sample B, thick suspension from bottom.	Control.	4.25	3.85	9.4
	Added methylene blue.		3.33	21.6

Warburg (17) in his studies on the respiration of goose erythrocytes produced hemolysis by repeated freezing and thawing. After prolonged centrifuging of this cytolyzed cell suspension at high speed two layers were formed—the upper containing the hemoglobin and fluid protoplasm, and the lower the solid cell particles, chiefly the nuclei. Respiration took place entirely in this lower layer. We have used Warburg's method to study glycolysis and methylene blue action in anemic goose blood hemolyzed by freezing and thawing. The clear supernatant fluid showed neither glycolysis nor oxidation by methylene blue, while the thick bottom layer showed both normal glycolysis and an increase of this process in the presence of methylene blue. The results of one such experiment are shown in Table X.

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Glycolysis and the action of methylene blue therefore seem to be intimately connected with the integrity of the cell structures. When the surface is damaged or destroyed the glycolytic process is slowed or ceases altogether, and with it, the oxidizing action of methylene blue.

Action of Methylene Blue upon Blood Serum and Glucose Solutions.

It has been known for a long time that glucose in alkaline solution can be oxidized by methylene blue. Spoehr (18) observed the

TABLE XI.

Action of Methylene Blue upon Glucose Solutions in Sørensen's Phosphate Mixtures (M/15) at pH 7.40 after Different Times of Incubation (37°).

	Glucose, mM per l.		Observations.
	Before incubation.	After incubation.	
4 hrs. incubation.....	9.26	9.23	No oxidation.
6 " "	8.26	8.26	" "
12 " "	10.91	10.94	" "

TABLE XII.

Action of Methylene Blue upon Glucose and Lactic Acid Content of Blood Serum. Incubation at 37° for 3 Hours.

	Glucose, mM per l.			Lactic acid, mM per l.		
	Before incubation.	After incubation.	Decrease.	Before incubation.	After incubation.	Increase.
Control.....	8.77	8.72	0	5.33	5.33	0
Added methylene blue.....		8.77	0		5.22	0

oxidation of sugar solutions with methylene blue in the presence of disodium phosphate in a continuous stream of air. The catalysis with methylene blue alone occurred only in alkaline solution, disodium phosphate being sufficiently alkaline for this purpose. It could be increased threefold when iron was added, which led him to assume that iron acted as a catalyst. The process described in the present paper is not related to Spoehr's observations as is indicated by the following experiments. Sørensen's phosphate mixture at pH 7.4, containing glucose, was incubated

at 37° for different lengths of time (4, 6, and 12 hours), methylene blue being added in the same concentration as was used in the experiments with blood. The amount of glucose after incubation was unchanged (see Table XI).

The action of methylene blue on serum containing glucose and lactic acid was then examined. Samples of serum were incubated for 3 hours at 37° with and without methylene blue. The glucose and the lactic acid remained unchanged (see Table XII). We conclude that methylene blue has no action upon glucose or lactic acid in the absence of the cells under these conditions.

Effect of Anaerobiosis in Relation to Glycolysis and Methylene Blue Action.

All of the experiments which are reported above have been performed under aerobic conditions. Owing to the method employed for defibrinating the blood and freeing it from leucocytes the material was completely saturated with oxygen just prior to its incubation. In the experiments in which dyes were added, it is true, the oxygen content of the material fell to a low level at the end of the experiment, owing to the increased oxygen consumption of the erythrocytes under such conditions, as was previously reported (1). In the following experiments, a comparison of glycolysis and lactic acid formation was attempted between completely reduced blood and blood which was kept completely and continuously oxygenated. A sample of blood was divided into four flasks, which were arranged in pairs connected with tubing in the thermostat at 38°. In one flask of each set methylene blue was added (0.005 per cent). The stoppers were sealed and a continuous slow stream of nitrogen was passed through one pair of flasks, while oxygen was passed through the other. The flasks were continuously agitated in a shaking device for 3 hours, at the end of which period the glucose and lactic acid contents were redetermined. (Table XIII.)

A constant stream of oxygen passing through blood undergoing glycolysis does not appear to affect this process appreciably as no difference was found between the samples exposed to the gas stream and control samples kept in stoppered flasks. On the other hand a stream of nitrogen which produced relative anaerobiosis increased glycolysis as well as lactic acid formation. In these

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TABLE XIII.

Action of Methylene Blue (M.B.) upon Blood Glycolysis and Lactic Acid Formation in Oxygenated and Reduced Blood. Incubation at 38° for 3 Hours.

Experiment No.		Blood glucose, mM per l.			Lactic acid, mM per l.			Calculated glycolysis in blood with M.B.	Oxidation by M.B., calculated as glucose.	Glycolytic quotient.
		Before incubation.	After incubation.	Decrease.	Before incubation.	After incubation.	Increase.			
								mM per l.	*mM per l.*	
	Goose blood.									
1	Oxygenated blood.									
	Control	8.48	6.11	2.37	5.87	7.07	1.20			0.26
	Added M.B		5.34	3.14		6.52	0.65	1.28	1.86	0.10
	Reduced blood.									
	Control		4.27	4.21		11.36	5.49			0.65
	Added M.B		4.21	4.27		10.68	4.81	3.70	0.57	0.55
2	Oxygenated blood.									
	Control	8.72	6.61	2.11	7.44	8.34	0.90			0.21
	Added M.B		4.96	3.76		8.03	0.59	1.38	2.38	0.08
	Reduced blood.									
	Control		4.90	3.82		12.00	4.56			0.60
	Added M.B		4.87	3.85		11.72	4.28	3.60	0.25	0.55
3	Oxygenated blood.									
	Control	7.55	6.00	1.55	4.66	5.37	0.71			0.23
	Added M.B		4.20	3.35		4.66			3.35	
	Reduced blood.									
	Control		4.45	3.10		8.43	3.77			0.61
	Added M.B		3.83	3.72		9.12	4.56	3.74		0.61
	Human blood.									
4	Oxygenated blood.									
	Control	6.17	3.96	2.21	2.70	7.10	4.40			1.00
	Added M.B		2.78	3.39		5.85	3.15	1.58	1.81	0.46
	Reduced blood.									
	Control		3.61	2.56		7.80	5.10			1.00
	Added M.B		3.17	3.00		7.80	5.10	2.56	0.44	0.85
	Dog blood.									
5	Reduced blood.									
	Control	8.16	6.16	2.00	2.78	6.22	3.44			0.85
	Added M.B		5.80	2.36		6.45	3.67	2.14	0.22	0.77

experiments it will be seen that the *glycolytic quotient* in the reduced blood, although higher than in the oxygenated blood, never reached unity, a fact which indicates that some oxidative process was going on while the blood was being reduced. It is known that muscle tissue in the presence of oxygen does not show a marked increase in lactic acid as this substance is in part oxidized and in part resynthesized to glycogen; under anaerobic conditions the amount of glucose destroyed corresponds to the lactic acid increase. In the present experiments the lactic acid formed in oxygenated goose blood is so low as to bring the *glycolytic quotient* down to 0.20, while in reduced blood (relative anaerobiosis) there is a rise of the lactic acid content which brings the *glycolytic quotient* up to 0.50 or 0.60. The similarity to the mechanism observed in muscle is evident.

Methylene blue, which produced its usual effect in the presence of oxygen, showed scarcely any oxidizing action upon the reduced blood. In one instance (Experiment 3, Table XIII) where nearly one-half of the glucose was oxidized in the presence of oxygen (no lactic acid increase), none was oxidized in the blood reduced by the nitrogen stream. It seems probable that all of the experiments would have shown similar results had strict anaerobiosis been attained. It is evident however that such a condition was not attained because the nitrogen used, while of high purity, contained on analysis traces of oxygen, which were also found in the blood on analysis at the end of the experiments.

In similar experiments with mammalian blood (Experiments 4 and 5, Table XIII) the flasks containing the samples were kept for 1 hour at 38° in a shaking device in a stream of pure nitrogen.[5] When the color of the blood indicated complete reduction, methylene blue was added. The flasks were then maintained under the same conditions for 2 or 3 hours. Even with these precautions oxidation by methylene blue was not completely stopped, but it was extremely small.

The failure of methylene blue to produce oxidation in blood undergoing glycolysis in the absence of oxygen is of some significance. A strong argument in favor of Wieland's theory is

[5] The highly purified nitrogen used in these experiments was prepared in an apparatus to be described shortly by Professor Michaelis to whose kindness we are indebted for the use of this material.

based upon Thunberg's (14) observation that methylene blue oxidizes succinic acid under anaerobic conditions. The presence of the oxyhemoglobin makes strict anaerobiosis difficult in material containing erythrocytes. The question is being studied with reference to the glycolysis produced by leucocytes. Of interest in relation to the effect of methylene blue upon erythrocyte oxidation under anaerobic conditions is the recent communication of Baumberger (19), who found that methylene blue at a concentration of 1 to 1,000,000 had a marked effect in postponing the onset of fatigue in frog muscle under anaerobic conditions. The oxygen equivalent of the methylene blue was far too small to account for the increased output of work produced. The changes in the lactic acid produced were not reported.

DISCUSSION.

It has been shown above that minute quantities of methylene blue in isotonic saline solution, when added to blood undergoing glycolysis, alter the carbohydrate metabolism in such a way that the velocity of sugar transformation is increased, while at the same time less lactic acid is formed. Coincident with this shift in the path of the metabolism there is a marked increase in oxygen consumption and carbon dioxide production, as shown in Paper I. We suggest that methylene blue produces its effect so far as carbohydrate is concerned by increasing the oxidation of some degradation product of glucose. This effect is not produced in blood in which normal glycolysis is absent, nor does it occur either in blood serum or in glucose solutions at pH 7.4. It is therefore not analogous to the oxidation of glucose by methylene blue in alkaline solutions as reported by other observers.

The point in the glycolytic process at which methylene blue exerts its action cannot be determined from our data. The chain of transformations which has been suggested by Shaffer (20) is well known: glucose → hexosephosphate → glyceric aldehyde → methylglyoxal → lactic acid.

Of these steps, the transformation of glucose to hexosephosphate, which does not require the presence of oxygen, and the splitting of glyceric aldehyde and methylglyoxal, which are transformed at once to lactic acid, seem to be excluded. Methylene blue has no effect on the oxidation of lactic acid in serum in which there is no

glycolysis. Where glycolysis does not occur in avian blood the addition of methylene blue produces no change in the concentration of lactic acid. Fürth and Lieben (21), however, have shown that lactic acid is consumed by horse erythrocytes, and Ray (22) working, it is true, at the unphysiological pH value of 6.9 has demonstrated the oxidation of lactic acid by dog erythrocytes. One cannot exclude the possibility that the observed methylene blue effect, at least in part, is due to oxidation of lactic acid.

In favor of the possibility that the principal point at which the methylene blue acts is upon the oxidation of hexosephosphate, is the fact that the action of methylene blue is increased by increasing the inorganic phosphate concentration, either alone, or together with added glucose. As to the exact nature of the methylene blue effect little may be said. It is conceivable that it acts as a coenzyme or catalyst, rendering the substrate (hexosephosphate?) more sensitive to the action of molecular oxygen. On the other hand one might consider that methylene blue plays in this system the rôle ascribed to iron in the oxidations produced by Warburg with his charcoal model.

CONCLUSIONS.

The relationship between glycolysis and the oxidative processes in blood cells may be expressed by the ratio

$$\frac{\text{Millimols of lactic acid increase}}{2 \times \text{millimols glucose decrease}}$$

which is termed the *glycolytic quotient.*

Normal adult mammalian red blood cells with almost no oxidative processes show a high *glycolytic quotient.* Nucleated erythrocytes (avian blood cells) with appreciable oxidative processes have a low *glycolytic quotient.*

The addition of methylene blue (or dyes with similar oxidation-reduction potentials) to the blood undergoing glycolysis produces an increased sugar degradation and a diminished formation of lactic acid. This is probably due in large part to the oxidation of some intermediary product. The *glycolytic quotient* is lowered.

The process begins only after the glucose molecule is acted upon by the glycolytic enzyme and is converted into other unstable and oxidizable substances. It is suggested that oxidation may

take place in the early stages of dissociation, before the glucose molecule is split into three carbon chain fragments, probably when the glucose molecule is esterified to hexosediphosphoric acid.

This reaction probably requires the presence of oxygen but the addition of cyanides does not impair the oxidative process. It is suggested that methylene blue acts as a catalyst, rendering the hexosephosphate molecule more sensitive to oxidation by molecular oxygen.

Our thanks are due to Professor Michaelis for his suggestions concerning a number of questions which have arisen in the course of this study.

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THE ELECTROMETRIC TITRATION OF HEMIN AND HEMATIN.

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It was shown 5 years ago (1) that the change from methemoglobin to hemoglobin could be studied by an electrochemical method. The reduction by means of sodium hydrosulfite and the oxidation of the reduced compound (hemoglobin) by ferricyanide were found to involve 1 equivalent. These results have been confirmed and extended in later work (2). An application of the same method to a study of the reduction of alkaline hematin solutions (1) yielded uncertain results which seemed to indicate that 2 equivalents of hydrogen were involved in the reduction of this compound. No definite conclusion could be drawn from the data, however. A continuation of this work has now shown that satisfactory electrochemical titration curves may be obtained *if titanous tartrate is employed as the reducing agent.* The results are very definite and show that the change involves *only 1 equivalent.*

While this work was in progress, two other workers reported results obtained by entirely different methods. These show that the reduction of hematin, like the reduction of methemoglobin, is the change from a ferric to a ferrous compound. The experiments which we now have to report, therefore, serve merely to confirm the recent observations of Haurowitz (3) and those of Hill (4).

Titration of Hemin and Hematin in Borate-Tartrate Buffer Solutions.

In Fig. 1 are shown typical titration curves of hemin solutions with titanous chloride in the presence of tartrate. The crystalline

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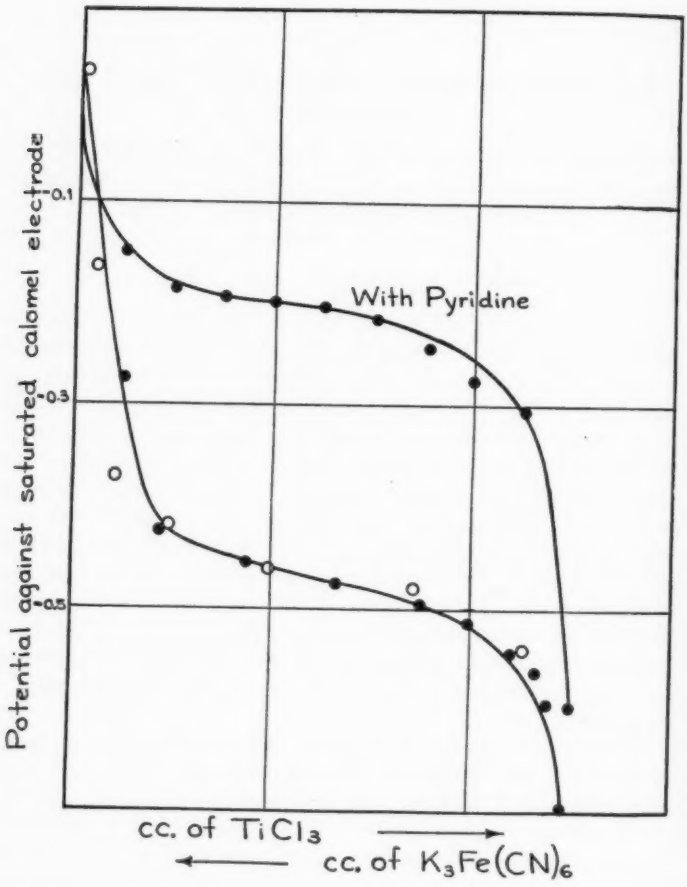

FIG. 1. Electrometric titration of hemin dissolved in borax-tartrate buffer solutions (pH 9.15). Clear circles indicate the points obtained in the back titration with ferricyanide.

hemin[1] was dissolved in 0.1 M borax either at room temperature or by warming, an equal volume of 0.2 M sodium tartrate was added, and the mixture placed in the usual electrochemical cell. After sweeping out all the air with oxygen-free nitrogen, dilute titanous chloride was added from a burette and the potential of two bright platinum electrodes noted. The potential was measured against a saturated calomel electrode. It was found necessary to use special precautions in purifying the nitrogen since the reduced hematin is extraordinarily sensitive to oxygen. Metallic nickel at a red heat or long trains of ammoniacal cuprous solutions were found to be fairly satisfactory.

The potentials are plotted vertically in Fig. 1 and the increments of reducing or oxidizing agent are plotted horizontally. The back titration with potassium ferricyanide is shown by clear circles. It is evident that the end-points are clearly defined. This is particularly true in the case of the titanous chloride titration in the presence of pyridine which causes a change of the position of the curve because the potential of the pyridine hemochromogen system is more positive than that of the reduced hematin system.

The presence of the tartrate ion in the alkaline buffer solution prevents the precipitation of titanous and titanic hydroxides. Thus, an alkaline complex titanous tartrate is in reality the reducing agent employed but, as solutions of it are very sensitive to oxygen, this indirect method of adding it was finally adopted. The addition of 3 per cent of mannite instead of sodium tartrate will also prevent the precipitation of titanous or titanic hydroxide but the titanous mannite complex does not have a sufficiently low potential to give a sharp end-point. The very dilute titanous chloride used was made by diluting the commercial 20 per cent material with boiled water; it was stored in an atmosphere of nitrogen. It slowly changed and was made up fresh every few days. It was standardized just before or after each titration by titrating electrochemically with potassium ferricyanide.

A few of the many results we have obtained are given in Table I

[1] The hemin used in this work was the α-hemin which was prepared by the usual acetic acid method from horse blood. It was purified by solution in chloroform and pyridine and reprecipitation in acetic acid containing sodium chloride. A few experiments with β-hemin prepared by Mörner's procedure showed that its reduction also required only 1 equivalent.

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which is self-explanatory. The numbers in the last column show that within the limits of the experimental error there is 1 mol of titanous chloride used in the reduction and 1 mol of ferricyanide in the reoxidation. The result is independent of the method of

TABLE I.

Determination of Number of Equivalents Involved in Reduction of Hemin or Hematin and Reoxidation of the Reduced Compound.

Experiment No.	pH	Iron compound.	Mols × 10^4.	Reagent employed.	Molality of reagent.	Amount of reagent.	Mols of reagent × 10^4.	Ratio of mols of reagent to mols of compound.
		mg.				*cc.*		
1	10.26	61.9	0.94	$TiCl_3$	0.0174	7.00	1.24	1.3
1 a	10.26	61.9	0.94	$K_3Fe(CN)_6$	0.0200	4.50	0.90	0.96
2	9.15	12.4	0.19	$TiCl_3$	0.0189	0.96	0.18	0.95
2 a	9.15	12.4	0.19	$K_3Fe(CN)_6$	0.0200	0.89	0.18	0.95
3	9.15	12.4	0.19	$TiCl_3$	0.0150	1.35	0.20	1.05
4	9.15	12.4	0.19	"	0.0125	1.95	0.24	1.3
5*	9.09	61.9	0.94	"	0.0167	6.00	1.00	1.06
5 a*	9.09	61.9	0.94	$K_3Fe(CN)_6$	0.02	5.00	1.00	1.06
6*	9.1	12.4	0.19	$TiCl_3$	0.0100	2.00	0.20	1.05
7*	9.1	12.4	0.19	"	0.0111	1.80	0.20	1.05
8	9.15	12.4	0.20	"	0.0140	1.40	0.20	1.0
9	9.15	12.4	0.20	"	0.0125	1.80	0.23	1.15

50 cc. of 0.1 M borax and 0.2 M sodium tartrate were used in each experiment; in those marked with an asterisk (*) pyridine was used also; sodium hydroxide was added in Experiment 1. Experiments 1 to 7 were performed with crystalline α-hemin, Experiment 8 with hematin prepared according to Küster (5), and Experiment 9 with dehydrochlorohemin. In Experiments 1, 5, and 7 the hemin was dissolved at room temperature; in the other experiments the iron compound was dissolved by warming it with the borax solution to 70° for 5 minutes, cooling to 20°, and adding the tartrate. The titrations with ferricyanide (Experiments 1 a, 2 a, 5 a) were performed with the solution reduced with $TiCl_3$ in the experiments of the same numbers.

dissolving the hemin and independent of the presence of pyridine. Hematin (Experiment 8) and dehydrochlorohemin (Experiment 9) also require 1 equivalent for the reduction. There can be no doubt from these results that the change in each case involves the reduction or the oxidation of the iron atom.

Aside from the information in regard to the number of equivalents involved in the oxidation or reduction, a satisfactory method of studying the iron-porphyrin compounds is of some interest, since it provides another method of characterizing these substances. This is of particular value in the case of the formation of easily dissociable complexes such as are formed between hemin and pyridine. Although the potentials are not as definite or as reproducible as in the case of the quinones, the method is useful even in extremely dilute solutions. For example, we have been able to obtain fairly satisfactory results in solutions of the pyridine-hemin compound which contained only 3×10^{-7} mols of hemin per liter. An electrochemical titration thus promises to be a useful supplement to the spectroscopic method of detecting and studying those iron-porphyrin derivatives which occur widely distributed in nature. We are now engaged in determining the oxidation-reduction potential of all the various iron-porphyrin compounds which can be obtained, including those formed by the decomposition of hemoglobin (α-hemin, β-hemin, "Verdauung's hematin," *etc.*) and their combinations with such substances as pyridine and the cyanide ion. The results of this work will be the subject of a later paper.

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THE ACTION OF ACETIC ANHYDRIDE AND PYRIDINE ON AMINO ACIDS.

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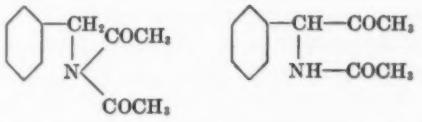
(Received for publication, June 26, 1928.)

In our first communication on this subject,[1] we reported the formation of unexpected derivatives of tyrosine and of phenylaminoacetic acid. In the early experiments with tyrosine, the crude reaction product appeared to have been obtained in a purer condition when the preparation was carried out in the presence of acetone. It therefore seemed possible that acetone took part in the reaction. Two possible structural formulæ were then given for the compound. However, in the experiment with phenylaminoacetic acid there was observed an abundant evolution of carbon dioxide. Hence, for this derivative two alternative formulæ were given.



It was definitely stated that the tyrosine derivative might have the same structure as that of phenylaminoacetic acid. In view of the lack of material and because of pressure of other work, the results have been published in a preliminary way. It was stated that the work on the details of the reaction was being continued.

It has since been found that the compounds described in the first paper are derivatives of acetylaminoacetone.

The constitution of the derivative from phenylaminoacetic acid was the first to have been established. On hydrolysis with hydrochloric acid it was converted into the corresponding hydro-

[1] Levene, P. A., and Steiger, R. E., *J. Biol. Chem.*, 1927, lxxiv, 689.

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chloride, which on treatment with alkali in the presence of oxygen of the air yielded a pyrazine. These compounds were identical with the hydrochloride of 1-phenyl-1-aminoacetone and with 3,6-dimethyl-2,5-diphenylpyrazine, both of which have been described previously. Analogous compounds were obtained from the tyrosine derivative.

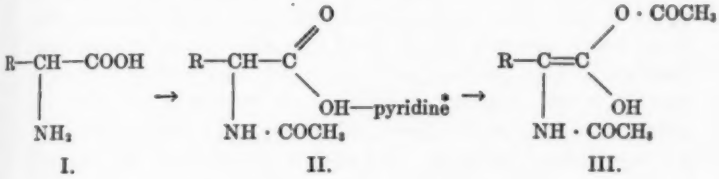
A uniform procedure was then adopted for preparing the acetylaminoketones. It consists of heating the amino acid (0.1 mol) with a mixture of equal weights of acetic anhydride[2] (100 gm.) and pyridine (100 gm.) in a bath maintained at 90° until the evolution of carbon dioxide has ceased.

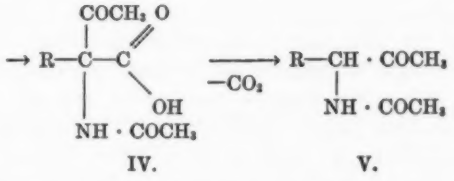
This procedure has been applied to phenylaminoacetic acid, phenylalanine, *l*-tyrosine, and phenylmethylaminoacetic acid. The carbon dioxide evolved during the reaction corresponded to 70 per cent of the theory in the case of the first three amino acids. The compounds obtained from *l*-tyrosine and phenylaminoacetic acid were identical with those described in our previous communication. A control experiment was performed in which phenylaminoacetic acid was heated with acetic anhydride in absence of pyridine. No loss of carbon dioxide occurred. In another experiment acetylphenylaminoacetic acid was used instead of the free amino acid, all other conditions being identical. The usual loss of carbon dioxide was observed. However, no carbon dioxide evolution was observed in the case of phenylmethylaminoacetic acid. Here the reaction product was the acetylated amino acid. The experiment is of great significance as it indicates the importance for the reaction of the presence of a mobile hydrogen atom on the carbon atom (2); it is also important as it shows that in the absence of such an atom even a mixed anhydride does not form. Of great importance in connection with this reaction is the fact that from the active tyrosine an inactive aminoketone is formed.

These observations lead us to believe that under the influence of pyridine, enolization occurs which permits the entry of an acetyl group in the molecule on the hydroxyl hydrogen of the carboxyl. The subsequent step consists of a migration of the

[2] Pure acetic anhydride (b.p. 139.46° under 760 mm.), obtained by a process developed by one of us (S.), was used throughout.

acetyl group to carbon atom (2); this step is then followed by carbon dioxide elimination with the formation of the ketone. The steps may be represented as follows:





(R = C_6H_5; $CH_3CO \cdot O \cdot (p) \cdot C_6H_4CH_2$; $C_6H_5 \cdot CH_2$)

Azlactones are probably formed as secondary products of the reaction.

When our work was completed, there appeared an article by Dakin and West,[3] who also have prepared acetylated aminoketones by the action of acetic anhydride on amino acids in the presence of pyridine. These authors have applied the method to a larger number of amino acids than we have done and also to other acids.

There is one point in the article of Dakin and West for which, at the present, we can give no explanation; namely, the discrepancy in the melting points of the substances $C_{12}H_{15}O_3N$ obtained on hydrolysis of the O-acetyl-N-acetylaminoketone derived from tyrosine. An error in the introduction of the article of Dakin and West wrongly attributes the discrepancy to the substance $C_{14}H_{17}O_4N$. The substance of this composition prepared by Dakin and West and the one prepared by us have the same melting point.

* The formation of a pyridine complex of unknown composition is assumed.

[3] Dakin, H. D., and West, R., *J. Biol. Chem.*, 1928, lxxviii, 91.

EXPERIMENTAL.

0.1 mol of amino acid was heated with a mixture of pure acetic anhydride (100 gm.) and dry pyridine (100 gm.) in a bath maintained at 90°. The carbon dioxide which escaped from the top of the reflux condenser was washed in a water scrubber and then absorbed in 1.0 N sodium hydroxide. The gas evolution ceased after 2½ hours. Carbon dioxide-free air was used to drive all carbon dioxide from the flask, condenser, and scrubber into the gas absorption apparatus. The carbon dioxide was determined by titration, phenolphthalein being used as an indicator. The reaction mixture was concentrated under reduced pressure to a thick syrup. It was twice reevaporated, each time with 25 cc. of xylene, in order to remove the residual pyridine and acetic anhydride. When the mixture was cooled and left to stand, crystallization took place. Purification of the crude products is best accomplished by crystallization from ketonic solvents (acetone, methylethylketone) despite the difficulties arising from the great solubility of the compounds in such solvents. The products were finally washed on the filter with ether. Further quantities of material were obtained from the mother liquors. When the hydrochlorides of the aminoketones are desired, the crude reaction product can be directly hydrolyzed with hydrochloric acid. Good yields of hydrochlorides are thus obtained. This procedure is more advantageous from a preparative point of view.

1-Phenyl-1-Acetylaminoacetone.—15.2 gm. of phenylaminoacetic acid yielded 19.2 gm. of crude crystalline product. It was purified by crystallization from 10 gm. of acetone. A first batch of crystals (8.3 gm.) melting at 100–101° was obtained.

No. 145.
$C_{11}H_{13}O_2N$ (191.17). Calculated. C 69.08, H 6.85, N 7.33.
Found. " 69.09, " 6.98, " 7.19 (Kjeldahl).

Molecular Weight Determination by Method of Menzies and Wright.—29.0 cc. of acetone (b.p. 56.5°, at 764 mm.); 0.6751 gm. substance; 22.5 mm. elevation on differential thermometer. Found 196. Calculated 191.2.

1-Phenyl-1-Aminoacetone Hydrochloride.—1. The crude crystalline reaction product (18.9 gm.) obtained on heating 19.4 gm. of

acetylphenylaminoacetic acid[4] with 95 gm. of acetic anhydride and 100 gm. of pyridine was refluxed for an hour with 200 cc. of 5.0 N hydrochloric acid. The dark solution, which had a very displeasing odor, was diluted with 200 cc. of water and 2 gm. of norit were added. When the filtrate was concentrated under reduced pressure, crystallization of the hydrochloride took place. The crystals were filtered off, washed with hydrochloric acid, then with ether, and dried in a vacuum desiccator over phosphorus pentoxide and soda-lime. The product (11.9 gm.) turned red at about 190° and melted around 200° with decomposition.

No. 141.

$C_9H_{12}ONCl$ (185.62).	Calculated.	N 7.55, Cl 19.10.
	Found.	" 7.28 (Kjeldahl), Cl 20.37 (Carius).

2. Acid hydrolysis was performed on pure 1-phenyl-1-acetylaminoacetone. 7.7 gm. (0.04 mol) were heated for an hour with 100 cc. of 5.0 N hydrochloric acid. The solution was diluted with an equal volume of water and finally evaporated to dryness under reduced pressure. The residue was dissolved in water, the solution decolorized with norit, and after addition of some hydrochloric acid, evaporated to dryness. Drying of the crystals was completed by their standing in a vacuum desiccator over phosphorus pentoxide and soda-lime. The product (6 gm.) turned red at about 190° and melted around 200° with decomposition.

No. 142.

$C_9H_{12}ONCl$ (185.62).	Calculated.	N 7.55, Cl 19.10.
	Found.	" 7.28 (Kjeldahl), Cl 20.19 (Carius).

When recrystallized from absolute alcohol, the hydrochloride analyzed as follows:

No. 137.

	Calculated.	N 7.55, Cl 19.10.
	Found.	" 7.54 (Dumas), Cl 19.04 (Carius).

It turned red at 200° and melted at 203°. The following data on this compound are recorded in the literature.

[4] Prepared according to the indications of Knoop, F., and Blanco, J. G., *Z. physiol. Chem.*, 1925, cxlvi, 273.

	Red coloration. °C.	M.p. °C.
Kolb.[5]	200	208
Gabriel.[6]	Around 190.	Around 200.
Emde.[7]	198–199	198–199

3,6-Dimethyl-2,5-Diphenylpyrazine.—1 gm. of 1-phenyl-1-aminoacetone hydrochloride was dissolved in 10 cc. of water. 0.35 cc. of ammonium hydroxide (*d* 0.90) was added. The precipitate which formed was washed with water and dried over phosphorus pentoxide. Yield 0.54 gm. It was dissolved in 4.5 gm. of boiling alcohol to which 1.8 gm. of water were then added. On being cooled crystals separated which were washed with 2.5 cc. of 70 per cent (by weight) alcohol. M.p. 124–125°. Kolb[8] found 124°, Gabriel,[9] 125–126°.

No. 140.
$C_{18}H_{16}N_2$ (260.24). Calculated. C 83.03, H 6.20, N 10.77.
Found. " 82.54, " 6.37 " 10.44 (Dumas).

1-Benzyl-1-Acetylaminoacetone.—16.5 gm. of phenylalanine yielded 21 gm. of crude crystalline product. It was recrystallized from acetone. A first fraction (7.9 gm.), melting at 95–96° was obtained. On recrystallization from acetone, the compound melted at 95–95.5°.

No. 146.
$C_{12}H_{15}O_2N$ (205.19). Calculated. C 70.21, H 7.37, N 6.83.
Found. " 70.56, " 7.72, " 6.71 (Kjeldahl).

1-Benzyl-1-Aminoacetone Hydrochloride.—The crude crystalline reaction product obtained from 16.5 gm. of phenylalanine was refluxed for an hour with 200 cc. of 5.0 N hydrochloric acid. The solution was shaken with 2 gm. of norit, then diluted with 200 cc. of water, and filtered. The filtrate was concentrated under reduced pressure to a thick syrup which had a very unpleasant odor. 10 cc. of xylene were added, and evaporation repeated in order to remove water. Crystallization then took place. This

[5] Kolb, A., *Ann. Chem.*, 1896, ccxci, 276.
[6] Gabriel, S., *Ber. chem. Ges.*, 1908, xli, 1152.
[7] Emde, H., *Arch. Pharm.*, 1909, ccxlvii, 134.
[8] Kolb, A., *Ann. Chem.*, 1896, ccxci, 277.
[9] Gabriel, S., *Ber. chem. Ges.*, 1908, xli, 1154.

residue was dissolved in 50 cc. of hot absolute alcohol, the solution filtered hot, and the hydrochloride precipitated by addition of 150 cc. of anhydrous ether. The crystals were filtered off after being cooled in ice, and washed with anhydrous ether. Yield 11.8 gm. M.p. = 123–125°.

No. 144.
$C_{10}H_{14}ONCl$ (199.63).
Calculated. N 7.02, Cl 17.76.
Found. " 6.88 (Kjeldahl), Cl 18.28 (Volhardt).

This product was not further purified.

3,6-Dimethyl-2,5-Dibenzylpyrazine.—2 gm. of 1-benzyl-1-aminoacetone hydrochloride were dissolved in 20 cc. of water. The oil precipitated on addition of 0.7 cc. of ammonium hydroxide (*d* 0.90) became solid after prolonged stirring. The product was washed with water and dried over phosphorus pentoxide. Yield 1.23 gm. It was dissolved in 4 gm. of boiling alcohol and 1 gm. of water was added; crystallization then set in. The crystals were washed with 50 per cent (by weight) alcohol and recrystallized from absolute alcohol. M.p. = 100–100.5°.

No. 149.
$C_{20}H_{20}N_2$ (288.28). Calculated. C 83.29, H 6.99, N 9.72.
Found. " 83.25, " 7.17, " 9.89 (Dumas).

1-(Acetyl-p-Hydroxybenzyl)-1-Acetylaminoacetone.—37 gm. (0.2 mol) of *l*-tyrosine, 200 gm. of acetic anhydride, and 200 gm. of dry pyridine were heated at 90° for 2½ hours. The solution was concentrated under reduced pressure to a syrup and the evaporation repeated three times, with addition each time of 50 cc. of xylene. The residue, which became solid on being cooled, was dissolved in 40 gm. of pure boiling methylethylketone. The solution was filtered while hot and the crystals deposited under cooling were washed on the filter first with a small amount of ketone, then with anhydrous ether. Yield 29 gm. A second fraction (6.5 gm.) was obtained from the mother liquor. These two fractions were combined and recrystallized from 40 gm. of methylethylketone. 24.1 gm., melting at 121–122.5°, were obtained. When recrystal-

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lized twice from double its weight of acetone, a product melting at 123–124° was obtained.

No. 150.
$C_{14}H_{17}O_4N$ (263.22). Calculated. C 63.85, H 6.51, N 5.32.
Found. " 64.21, " 6.76, " 5.19 (Kjeldahl).

This compound was optically inactive.

1-(p-Hydroxybenzyl)-1-Aminoacetone Hydrochloride.—2.7 gm. (0.01 mol) of pure acetylated aminoketone from tyrosine were refluxed for 1 hour with 30 cc. of 5.0 N hydrochloric acid. The solution was then diluted with 30 cc. of water and evaporated to a very thick syrup which crystallized on standing in a desiccator over phosphorus pentoxide and soda-lime. This residue, after being stirred with anhydrous ether, absolute alcohol, and petrolic ether, was filtered. It was finely powdered and digested and washed with ether. Yield 1.4 gm. M.p. = 165–166° with decomposition.

No. 143.
$C_{10}H_{14}O_2NCl$ (215.63).
Calculated. N 6.50, Cl 16.45.
Found. " 6.16 (Kjeldahl), Cl 16.70 (Carius).

The compound was not further purified.

3,6-Dimethyl-2,5-Di-(p-Hydroxybenzyl)-Pyrazine.—1.08 gm. (0.005 mol) of aminoketone hydrochloride were dissolved in 5 cc. of water. On addition of 0.35 cc. of ammonium hydroxide (*d* 0.90), a solid precipitate was obtained, which was stirred and washed with water and dried over phosphorus pentoxide. Yield 0.7 gm. It was crystallized from a water-pyridine mixture.

No. 153.
$C_{20}H_{20}N_2O_2$ (320.28). Calculated. C 74.96, H 6.30, N 8.75.
Found. " 74.98, " 6.41, " 8.84 (Dumas).

Acetylphenylmethylaminoacetic Acid.—16.1 gm. of phenylmethylaminoacetic acid gave 19.6 gm. of crude crystalline product. It was recrystallized from 30 gm. of acetone. The crystals which deposited were washed with anhydrous ether. They were united with the successive fractions obtained by concentrating the mother liquors. The combined product melted at 190–193°.

2 gm. were recrystallized from 7 gm. of boiling absolute alcohol in the presence of some norit. The crystals were washed with ether. M.p. = 192–193.5°.

No. 148.
$C_{11}H_{13}O_3N$ (207.17).
Calculated. C 63.75, H 6.32, N 6.76.
Found. " 64.02, " 6.45, " 6.55 (Kjeldahl).
" 0 (Van Slyke).

Phenylmethylaminoacetic Acid.—The preceding compound yielded this amino acid on hydrolysis. 2.1 gm. of acetylphenylmethylaminoacetic acid were refluxed for 1 hour with 30 cc. of hydrochloric acid. The solution was diluted with 30 cc. of water and evaporated under reduced pressure to complete dryness. The solution of this residue in 15 cc. of water was decolorized with norit and made slightly alkaline with ammonium hydroxide. On addition of alcohol the amino acid crystallized. It was washed with water, alcohol, then with ether, and dried at 100°.

No. 151.
$C_9H_{11}O_2N$ (165.15). Calculated. C 65.42, H 6.72, N 8.48.
Found. " 65.73, " 6.45, " 8.33 (Kjeldahl).

SUMMARY.

1. The action of acetic anhydride and pyridine on phenylaminoacetic acid, phenylalanine, and *l*-tyrosine has been studied. The main products of the reaction are carbon dioxide and derivatives of acetylaminoacetone of the formula $R–CH·(NH·COCH_3)·COCH_3$. With phenylmethylaminoacetic acid no loss of carbon dioxide occurs; simple acetylation takes place.

2. The constitution of the ketonic compounds has been established by their conversion into the corresponding hydrochlorides and pyrazines.

3. A reaction mechanism is suggested to explain the observed facts.

COMPOSITION OF BONE.

I. ANALYTICAL MICRO METHODS.*

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(Received for publication, June 2, 1928.)

There are no methods in the literature for the quantitative estimation of the inorganic constituents of very small amounts of calcified material. Kramer and Howland (1) recently described improved methods of bone analysis, but their procedure calls for the use of 250 to 500 mg. of bone powder for each determination of carbonate.

With the technique described here a single determination of carbonate is performed on about 20 mg. of bone powder; 50 mg. suffice for the determination in duplicate of carbonate, calcium, and inorganic phosphorus. These three determinations, in duplicate, have been performed in some instances on half this amount of bone powder. This has enabled us to analyze minute specimens, such as the head bones of a human fetus which when dried and pulverized weighed 17.7 mg., and the calcified cusp of an aortic valve which gave 21.3 mg. of bone powder.

Preparation of Material.

The bones (or other calcified tissues) were removed from the body immediately after death. The adherent soft tissues were dissected from the bones which were then crushed into small fragments. The crushed bones were immediately extracted in a Soxhlet extraction apparatus with a mixture of ethyl alcohol and ether for about 4 hours. Before beginning the extraction this mixture was made alkaline with NaOH. Destruction of bone

* A preliminary report was presented at the Twenty-First Annual Meeting of the American Society of Biological Chemists, April, 1927 (*J. Biol. Chem.*, 1927, lxxiv, p. ix).

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carbonate during the extraction because of contact with volatile acids was thus avoided since only the condensed vapors from the alkaline mixture came into contact with the bones.

The alcohol and ether were removed from the extracted bones by evaporation at a temperature not exceeding 38°. The bones were then pulverized finely and passed through an 80 mesh sieve. The preparation of the material for analysis was completed within 24 hours after removal of the specimen from the body.

Determination of Carbonate.

Carbonate was determined in Van Slyke's (2) manometric blood gas apparatus. Some difficulty was experienced in the introduction of the sample, for the particles of bone powder tended to adhere to the shoulder at the bottom of the cup (see (2), Fig. 4). The construction of the cup and of the stop-cock just below the cup was therefore modified; these modifications facilitated the complete introduction of the bone powder. The shoulder and capillary at the bottom of the cup were eliminated, and instead of a stop-cock with a 1 mm. bore, we employed one in which the bore tapered from 4 mm. at the upper end to 2 mm. at the lower end. The rest of the apparatus was of standard design.

For analysis sufficient bone powder was taken to liberate about 100 mm. of CO_2 as read on the closed manometer scale. In general, about 20 mg. sufficed. The sample was weighed on a balance weighing to 0.05 mg., a small piece of lead foil (4 cm. × 3 cm.) being used instead of weighing paper. Weighing paper was found unsatisfactory for this purpose, since it was observed that its weight occasionally fluctuated. The cause of these fluctuations was not determined; they may have been due to changes in moisture content or, more probably, to electrostatic forces.

About 0.5 cc. of mercury was introduced at the bottom of the cup and 2 drops of distilled water were placed on top of the mercury. The bone powder was then poured into the cup. No attempt was made to remove the powder completely from the lead foil, since the weight of the sample was obtained by difference after weighing the empty lead foil.

When properly introduced, most of the powder fell on the water at the base of the cup; very little or none adhered to the upper wall. By suitable manipulation, the powder was drawn

into the 50 cc. chamber. Care was taken to keep the powder in the upper half of this chamber. 2 or 3 cc. of mercury were run into the cup; then 2 drops of water were placed on top of the mercury and the level of this column was slowly lowered until the cup was empty. This method of washing reduced greatly the amount of water necessary for washing in the sample since the column of mercury with a thin film of water on top was equivalent to an equal column of water. The washing was continued until no particles of powder were visible on the sides of the cup. About 1 cc. of water generally sufficed when it was used a drop or two at a time in this way.

After the sample was washed in, the level of the water was raised almost to the upper stop-cock to expel most of the air from the chamber. It was considered unnecessary to remove all the air above the solution since the CO_2 evolved was determined by absorption with alkali. The dissolved gases in the 1 cc. of water were not removed; this was taken care of by means of a blank analysis. 7 cc. of approximately 3.5 N H_2SO_4 were poured into the cup, and 5 cc. admitted to the chamber, the remaining 2 cc. of acid serving as a seal. Mercury was then run into the cup, displacing the 2 cc. of acid. Enough of this mercury was admitted to fill the bore of the stop-cock and seal the chamber. The acid remaining in the cup was then removed. After the addition of the acid, the mercury in the chamber was lowered almost to the 50 cc. mark. The acid was then allowed to remain in contact with the powder for about 30 minutes or until no more bubbles of CO_2 were liberated. The contents of the chamber were then shaken for about 5 minutes and the pressure read at the 2 cc. mark. The level was again lowered, shaking was continued for 2 minutes, and the pressure again read. This was repeated until all the CO_2 had been liberated as evidenced by no further increase in pressure. Usually after the second or third shaking a constant pressure (p_1) was obtained. 5 cc. of approximately 13 N KOH were now poured into the cup. 2 cc. were admitted to absorb the CO_2, and the upper stop-cock sealed with mercury as described above. As a result of the reaction between the strong acid and base, considerable heat developed. The solution was, therefore, shaken for 2 minutes to obtain a uniform temperature. The pressure was then read again (p_2).

Calculations.—It is possible to calculate the CO_2 content of the

bone directly from the gas laws. The volume of the gas is 2 cc., the pressure is the difference between p_1 and p_2, and the temperature is taken as the mean of the two temperatures before and after absorption. Since the difference in the two temperatures is about 1°, no great error is thus introduced. From the volume of evolved CO_2, reduced to 0° and 760 mm., and from the weight of the sample, the per cent of CO_2 is readily calculated.

It is, however, more convenient to utilize the tables prepared by Van Slyke and Sendroy (3). When the per cent of CO_2 is calculated from the following formula the same result is obtained as is given by direct calculation from the gas laws.

$$\frac{\text{Mm. pressure} \times \text{factor} \times 4.4}{\text{Weight of sample in mg.}} = \text{per cent of } CO_2$$

The pressure $= p_1 - p_2 - c$ where p_1 and p_2 are the pressures in mm. before and after absorption and c is the blank. The blank used was the value obtained by performing the operations described above without the use of bone powder. The difference in pressure before and after absorption in the blank analyses is the correction c; this amounted to about 3 mm.

The factor employed is that taken from Van Slyke and Sendroy (3) (the last column of Table X, p. 142). In selecting the factor, the average temperature is employed as stated above. It can readily be shown that the above convenient expression is theoretically valid, and that it gives the same values as direct calculation.

Determination of Calcium and Phosphorus.

Again determined by difference, about 10 mg. of bone powder were weighed and transferred to a dry 10 cc. volumetric flask. 2 cc. of 1 M HCl were added and the mixture was then digested in a boiling water bath for about 10 minutes. When cool, 3 cc. of 20 per cent trichloroacetic acid were added and the volume was made up to 10 cc. with water. The solution was then filtered through a small funnel with a diameter at the top of about 2.5 cm. Munktell's filter paper No. 00 was used. To insure the absence of acid-extractable material, the filter paper was washed freely, first with HCl and then with water. When dry it was cut down to the desired size.

Calcium was determined in duplicate on 2 cc. aliquots of this filtrate according to the method of Kramer and Tisdall. The pH of the solution was adjusted with brom-cresol purple after addition of the ammonium oxalate.

The filtrate from the bone solutions was often turbid. A number of methods for removing the turbidity were tried without success. The following procedure was finally found to give a clear filtrate in every instance.

A 2 cc. aliquot of the first filtrate was transferred to a 10 cc. volumetric flask, and 5 cc. of molybdic acid reagent were added. This reagent is the one employed in the Briggs-Bell-Doisy method for phosphorus. The volume was made up to the mark with water. After standing for about half an hour, it was filtered in the same fashion as the first solution. For the phosphorus determination, 4 cc. aliquots of this second filtrate were used. To each of two 4 cc. aliquots in a 10 cc. volumetric flask were added 1 cc. of sulfite and 1 cc. of hydroquinone according to the Briggs-Bell-Doisy method. The volume was made up to 10 cc. and the color compared with the standard.

At the beginning of the investigation, if the first filtrate was clear, phosphorus was determined directly on 2 cc. aliquots. It frequently happened that some clear filtrates subsequently developed a turbidity during the development of the blue color. This turbidity seriously interfered with the accuracy of the results. The addition of the molybdic acid reagent and the second filtration were therefore adopted as a routine procedure whether the first filtrate was clear or not.

In making up the standard comparison solutions the same amounts of trichloroacetic acid and hydrochloric acid were added to the known solution as were contained in the aliquots of the unknown. In this way errors due to differences in pH and to the effect of organic substances in the reagents as pointed out by Stanford and Wheatley (4) were ruled out. The standard solution employed contained 0.025 mg. of P per cc. Usually 2 cc. were taken for comparison. When the phosphorus content in a particular specimen is high, more of the standard solution may be used or smaller aliquots of the bone solution may be taken. In order to obtain accurate results with this technique the amount of phosphorus in the unknown and in the standard should not differ by more than about 30 per cent.

TABLE I.

Reproducibility of Acid Digestion.

Bone.	Sample.	Ca	P	$\frac{\text{Residual Ca}}{\text{P}}$.
		per cent	*per cent*	
A	a	24.6	10.6	
		25.2	10.7	1.98
		24.9	10.7	
	b	23.9	10.2	
		24.2	10.1	2.00
		24.1	10.2	
	c	24.6	10.6	
		24.9	10.5	1.99
		24.8	10.6	
B	a	13.7	6.0	
		13.2	6.0	2.05
		13.5	6.0	
	b	13.4	6.4	
		13.2	6.2	1.92
		13.3	6.3	
C	a	12.4	5.7	
		12.8	5.6	2.02
		12.6	5.7	
	b	13.2	6.1	
		13.0	5.9	2.00
		13.1	6.0	
D	a	19.7	8.8	
		20.1	8.8	2.02
		19.9	8.8	
	b	19.2	8.5	
		19.4	8.7	2.00
		19.3	8.6	
E	a	24.0	10.1	
		24.5	10.2	2.04
		24.3	10.2	
	b	24.6	10.2	
		23.3	10.0	2.03
		24.0	10.1	

Reproducibility of Acid Digestion.

Table I gives the reproducibility obtained by the acid digestion described above. Three 10 mg. samples of Bone A were digested separately in an identical manner and then analyzed, the calcium

TABLE II.

Effect of Molybdate Precipitation on Phosphorus Content.

Bone.	Filtrate.	P	Residual Ca / P.
		per cent	
I	1	14.3	
		14.3	1.82
		14.3	
	2	13.2	
		13.2	1.98
		13.2	
II	1	7.0	
		6.8	1.86
		6.9	
	2	6.5	
		6.7	1.94
		6.6	
III	1	11.7	
		11.9	1.76
		11.8	
	2	10.6	
		10.4	1.98
		10.5	
IV	1	11.8	
		11.6	1.80
		11.7	
	2	10.3	
		10.5	2.04
		10.4	

Ca:P in $Ca_3(PO_4)_2$ = 1.94.

and phosphorus in each sample being determined in duplicate. The agreement among three samples digested separately is about the same as that between aliquots of the same solution. Similar results were obtained with Bones B, C, D, and E.

The question arose as to whether the precipitate caused by the addition of the molybdic acid reagent to aliquots of the first filtrate adsorbed some of the inorganic phosphate. If this were the case, then erroneous values for the ratio residual Ca:P would be obtained. To see whether this occurred several normal bones were analyzed for calcium and CO_2 in duplicate as described above

TABLE III.

Comparison of Cold Digestion and Hot Digestion.

Bone.	Digestion.	Ca	P	$\frac{\text{Residual Ca}}{\text{P}}$.
		per cent	*per cent*	
A	Hot.	23.9	10.2	
		24.2	10.1	2.00
		24.1	10.2	
	Cold.	24.1	10.3	
		23.9	10.2	1.97
		24.0	10.3	
E	Hot.	24.0	10.1	
		24.5	10.2	2.04
		24.3	10.2	
	Cold.	23.5	10.1	
		23.4	10.2	1.96
		23.5	10.2	
F	Hot.	20.1	9.1	
		20.4	8.6	2.01
		20.3	8.7	
			8.8	
	"	20.2	8.6	
		20.7	8.7	2.06
		20.5	8.7	
	Cold.	20.8	8.9	
		20.8	8.9	2.04
		20.8	8.9	

while phosphorus was determined on aliquots of both the first and second filtrates in each case. The final blue phosphate solutions obtained from the second filtrates were always clear, while those from the first filtrates were more or less turbid. The former gave excellent color matches with the standard, while the latter were more or less off color. The results are given in Table II. Ratios

calculated from the second filtrate are 1.98, 1.94, 1.98, and 2.04 while from the first filtrate they are 1.82, 1.86, 1.76, and 1.80. In normal bone the ratio is about 1.94 according to Howland, Marriott, and Kramer (5). These results show that the second precipitation and filtration do not remove sufficient inorganic phosphorus to give erroneous values for the ratio.

When the molybdate reagent is added to an aliquot of the first filtrate, the precipitate that forms on standing is flocculent and appears large in amount. It was found that cold acid digestion greatly diminished the amount of organic material that went into solution. When the bone solutions were prepared according to the technique described previously except that the digestion with acid was carried out by shaking in the cold, very little organic material dissolved. This was shown by the very small amount of precipitate subsequently obtained when the molybdate reagent was added. The same analytical results were obtained with both hot and cold acid digestions as is shown in Table III.

However, when cold digestion was used the amount of precipitate produced by molybdate was so small in some cases that it did not coagulate, and on filtration, passed into the filtrate. This turbidity in the second filtrate interfered with the phosphate analysis. Hot digestion was therefore used as a routine, as it invariably resulted in a clear second filtrate.

Effect of Delaying Ether-Alcohol Extraction.

The length of time elapsing between removal of the specimen from the body and the completion of the preparation for its analysis is another possible source of error. This of course is a matter not peculiar to the micro technique, but to any method of bone analysis. If the bones, after removal from the body, are permitted to stand for some time before the extraction with ether and alcohol, it is possible that some of the carbonate may be decomposed, and some of the CO_2 lost. There is also the possibility to be considered that the percentage of inorganic phosphate may undergo some change.

Accordingly, a quantity of normal bone, Bone A, was crushed and divided into two portions immediately after removal from the body. One portion was extracted, dried, and pulverized on the day of autopsy. The other portion was put into a cork-stoppered

bottle and was set aside for 5 months. It remained in this bottle at room temperature throughout the warmest part of the year, from April to September. After 5 months it was extracted and prepared for analysis in the usual manner.

The results of the analysis are given in Table IV. It is seen that the content of calcium, inorganic phosphorus, and CO_2 had not altered materially as a result of this long delay. Bone E is another specimen of normal bone which was tested in the same way with similar results. In these two instances standing unextracted for months did not materially affect the composition.

TABLE IV.

Effect of Delaying Ether-Alcohol Extraction.

Bone.	Time extracted.	Ca	P	CO_2	$\frac{\text{Residual Ca}}{\text{P}}$.
		per cent	*per cent*	*per cent*	
A	Immediately.	24.6	10.6	4.05	
		25.2	10.7	4.16	1.98
		24.9	10.7	4.11	
	After 5 mos.	24.9	10.3	4.07	
		24.8	10.5	3.99	2.04
		24.9	10.4	4.03	
E	Immediately.	24.0	10.1	3.79	
		24.5	10.2	3.86	2.04
		24.3	10.2	3.83	
	After 5 mos.	24.1	10.6	3.90	
		24.7	10.4	3.94	1.98
		24.4	10.5	3.92	

This was contrary to our expectations. It may well be, however, that other kinds of bones, or even the same bones, under somewhat different conditions may undergo considerable change if they are allowed to stand unextracted.[1] To avoid this possibility, we

[1] In one instance we did obtain abnormal results. Fragments of rat bone were allowed to stand in an open dish for several months. Analysis of this material gave

Ca *per cent*	P *per cent*	CO_2 *per cent*	
10.7	4.0	1.79	$\frac{\text{Residual Ca}}{\text{P}} = 2.35$
11.0	4.0	1.42	
10.9	4.0	1.61	

adopted the policy of extracting the calcified specimen immediately after removing it from the body.

Reproducibility of CO_2 Analyses.

Table V gives the results for CO_2 on various calcified tissues containing different amounts of CO_2. The greater the CO_2 content the smaller of course will be the sample required to liberate 100 mm. of CO_2 in the closed manometric apparatus. Samples much smaller than 5 mg. should not be used as then an error of 0.1 mg. in weighing will give more than a 2 per cent error in the weight of the sample. The reproducibility of the CO_2

TABLE V.

CO_2 Analysis of Bones.

Specimen.	Weight of sample.	CO_2	Specimen.	Weight of sample.	CO_2
	mg.	*per cent*		*mg.*	*per cent*
G	21.6	1.83	J	10.8	4.66
	21.7	1.88		8.9	4.70
H	21.7	2.33	K	18.8	3.02
	23.0	2.13		6.5	3.03
				6.6	2.95
I	10.5	3.63	L	6.2	2.60
	11.2	3.69		4.3	2.56

determination was as good as those of the calcium and phosphorus determinations.

Ratio, Residual Ca:P.

The ratio, residual Ca:P, was calculated in a manner similar to that of Howland, Marriott, and Kramer (5). The per cent of CO_2 multiplied by 0.91 gave the per cent of calcium assumed to be present as carbonate calcium. The residual calcium was obtained by subtracting the carbonate calcium from the total calcium, all expressed in per cent or gm. per 100 gm. of bone. Thus

$$\frac{\text{Residual Ca}}{\text{P}} = \frac{\text{(total Ca)} - \text{(carbonate Ca)}}{\text{total inorganic P}}$$

Table VI is a summary of the ratios obtained in forty-four analyses performed with this micro technique. The mean obtained was 2.00 ± 0.01. The value for *a.d.*, the average deviation, was obtained in the usual way; *A.D.* has its usual signifi-

TABLE VI.

Summary of Ratios.

$\frac{\text{Residual Ca}}{\text{P}}$.	Deviation.	$\frac{\text{Residual Ca}}{\text{P}}$.	Deviation.
1.98	−0.02	1.96	−0.04
1.99	−0.01	1.97	−0.03
2.00	0.00	1.97	−0.03
1.97	−0.03	1.90	−0.10
2.04	+0.04	1.88	−0.12
2.04	+0.04	2.12	+0.12
2.03	+0.03	2.00	0.00
1.96	−0.04	2.02	+0.02
1.98	−0.02	2.21*	+0.21
1.91	−0.09	2.02	+0.02
1.91	−0.09	2.00	0.00
2.01	+0.01	2.08	+0.08
1.86	−0.14	1.93	−0.07
1.95	−0.05	2.12	+0.12
1.96	−0.04	2.12	+0.12
1.98	−0.02	1.94	−0.06
1.89	−0.11	2.05	+0.05
1.95	−0.05	1.92	−0.08
2.05	+0.05	2.10	+0.10
1.82	−0.18	2.00	0.00
		2.14	+0.14
		2.10	+0.10
		2.03	+0.03
		2.01	+0.01
		Mean = 2.00	*a.d.* = ±0.06
			A.D. = ±0.01

Residual Ca:P = 2.00 ± 0.01. Ca:P in $Ca_3(PO_4)_2$ = 1.94.

* Two other analyses of the same bone gave ratios of 2.02 and 2.00.

cance, *i.e.* $A.D. = \frac{a.d.}{\sqrt{n}}$. An obviously erroneous value of 2.21 was included for the sake of completeness although two other analyses, each in duplicate, of the same specimen of bone powder

gave ratios of 2.02 and 2.00. This table contains all the ratios which we have obtained on normal rat bone; none has been omitted. It also contains all the ratios which we have obtained on pathologically calcified human tissues with the exception of the three ratios given in Table II of the following paper (6). The ratios in this table exhibit the normal distribution of deviations about a mean; very high values and very low values are rare. The mean value of 2.00 ± 0.01 does not differ widely from 1.94, the Ca:P value usually given for normal bone; it is about 3 per cent higher than the theoretical value of 1.94 for $Ca_3(PO_4)_2$.

DISCUSSION.

The numerator of our ratio is the same as that of Howland, Marriott, and Kramer; *i.e.*, residual Ca is the per cent of calcium remaining after the carbonate calcium is deducted from the total calcium. For the denominator we have used the total amount of inorganic phosphorus. This is different from the calculation of Howland, Marriott, and Kramer, who use as the denominator the residual phosphorus obtained by deducting from the total phosphorus the amount calculated as bound to magnesium in the compound $Mg_3(PO_4)_2$.

These authors showed ((5), p. 724) that for a typical bone, the per cent of magnesium present was 0.24. If this is all present as $Mg_3(PO_4)_2$, then the per cent of phosphorus in this bone present as magnesium phosphate is 0.3. For an error of ±3 per cent in the phosphorus determination, they found for this bone that the total phosphorus in per cent was 11.6 ± 0.35. Thus the amount of phosphorus bound as magnesium phosphate is about the same as the error in the determination of total inorganic phosphorus.

This probably accounts for the fact that we obtained normal ratios even though the magnesium analysis was omitted. With very small specimens, which do not contain enough material for a complete analysis, omission of the magnesium determination is therefore preferable to omission of any of the other three.

For the CO_2 analysis enough sample was taken to obtain about 100 mm. of CO_2. As stated previously, about 20 mg. of bone powder were usually sufficient. When only about 20 mg. were available for the determination of CO_2 we preferred to make the analysis in duplicate using half of the available quantity for each

analysis rather than rely on a single analysis. Of course, with half the stated quantity of CO_2, the relative error is doubled since the absolute errors remain the same. The Ca:P ratio, however, is not markedly affected even when the error in CO_2 is doubled, for the error in residual Ca is increased by only about one-fifth of the increase in the error in CO_2.[2]

The occurrence of turbidity immediately after addition of molybdic acid or during the development of the color in the colorimetric determination of phosphorus has been observed by Bell and Doisy (7), Briggs (8), Benedict and Theis (9), and Fiske and Subbarow (10). The turbidity was ascribed to the precipitation of some unknown substance or substances present in urine and blood. Briggs suggested that the precipitate may be due to the interaction between molybdic acid and a small amount of protein which escaped precipitation because of insufficient shaking after the trichloroacetic acid was added. To avoid the turbidity he recommended vigorous shaking and a delay of 10 minutes before the precipitated proteins were filtered off. Fiske and Subbarow were of the opinion that a small amount of protein escaped precipitation as a result of too low a concentration of trichloroacetic acid, and was subsequently precipitated by phosphomolybdic acid. They recommended an increase in the concentration of trichloroacetic acid.

The above observations were made on filtrates from blood and urine. As far as bone solutions are concerned, the nature of the precipitate which forms on addition of molybdic acid is unknown. The amount of precipitate formed on addition of trichloroacetic

[2] In adult rats $\frac{\text{carbonate Ca}}{\text{total Ca}} = 15$ per cent approximately, therefore, when total Ca = 28.00 per cent then carbonate Ca = 4.00 per cent and residual Ca = 24.00 per cent. When the error in the CO_2 analysis is

	2 per cent *per cent*	4 per cent *per cent*
Total Ca	= 28.00	= 28.00
Carbonate Ca	= 4.08	= 4.16
Residual Ca	= 23.92	= 23.84

Thus an error of 2 per cent in the CO_2 causes an error in residual Ca of 1 part in 240 or about 0.4 per cent; and a 4 per cent error in CO_2 gives an error of 2 parts in 240 or about 0.8 per cent in residual Ca, *i.e.* only about one-fifth the error in CO_2.

acid to the original bone solution is small; it is very much less in amount than that obtained in similar procedures with blood. Upon addition of molybdic acid to the first filtrate, variable amounts of turbidity and precipitate are obtained from the same bone powder, depending upon the temperature at which the bone was digested with HCl. This points to the presence in the filtrate of intermediate products of protein decomposition. In this connection it is of interest to note that Hiller and Van Slyke (11) found that while trichloroacetic acid precipitated proteins, it permitted intermediate products such as "proteoses" and "peptones" to pass into the filtrate.

In any case the same phosphorus values and normal Ca:P ratios were obtained whether the molybdate precipitate was comparatively large in amount (as in the hot digestions) or very much smaller in amount (as in the cold digestions). This can be seen from Table III. Thus the amount of phosphate removed by this precipitate, whether by chemical combination or by adsorption, appears either to be negligible or to be within the limit of error of the method used to determine phosphorus.

The mean value of 2.00 ± 0.01 is somewhat higher than the ratios obtained by Howland, Marriott, and Kramer. It is near enough, however, to their value to be regarded as satisfactory for a micro technique.

While these procedures have been developed to permit analysis of very small specimens of calcified tissues, they may also be used for determining CO_2, calcium, and phosphorus in solids other than bone.

SUMMARY.

1. A technique is described for determining the CO_2 content of bone for which only about 20 mg. of bone powder are required.

2. A technique is described for determining in duplicate both calcium and inorganic phosphorus in bone in which only about 10 mg. of bone powder are required.

3. With the procedures outlined here, CO_2, calcium, and inorganic phosphorus in bone may be determined in duplicate on about 50 mg. of material; the entire procedure has been performed on as little as 17.7 mg.

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COMPOSITION OF BONE.

II. PATHOLOGICAL CALCIFICATION.*

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By means of the micro technique described in the preceding communication (1) we have analyzed a number of minute specimens of pathological calcification obtained from humans. The analytical results are given in Tables I and II.

The last column in Table I shows that these specimens have normal ratios; *i.e.*, the same Ca: P ratio, within the experimental deviation, as is found in normal bones.

One type of pathological calcification, however, seems to have a composition different from normal bone. Table II gives the analytical results for three specimens of calcified fibroid of the uterus obtained from three different individuals. The ratios 2.23, 2.23, and 2.18 do not appear to be attributable to experimental error. In the case of fibroid Specimen A, there were two kinds of calcification in the specimen: one a chalky deposit, "chalk," and the other a tough, firm deposit, "hard." These were analyzed separately. The former gave a normal value of 1.98, while the latter gave an abnormal value of 2.23.

In his Harvey Lecture for 1911 Wells (2) stated that the composition of pathological calcific deposits is the same as that of normal bone. Recently Wells (3) again stated: "The composition of the inorganic salts in calcified areas in the body seems to be practically the same, if not identical, whether the salts are laid down under normal conditions (ossification) or under patho-

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logical conditions." The only instance in which it was reported that the ratio of Ca: P was different from that of normal bone was that given by Eden (4). Eden found that in young callus the ratio Ca: P was greater than in mature bone, and that as the callus became older and was replaced by true bone the ratio

TABLE I.
Pathological Calcification (Human).

Specimen.	Ca	P	CO_3	$\frac{\text{Carbonate Ca}}{\text{Total Ca}}$.	$\frac{\text{Residual Ca}}{\text{P}}$.
	per cent	*per cent*	*per cent*	*per cent*	
Thyroid.	20.1	9.1	3.01		
	20.2	9.1	3.18	14	1.91
	20.2	9.1	3.10		
Tuberculous lymph nodes.	31.7	15.0	4.66		
	32.9	14.7	4.70	13	1.88
	32.3	14.9	4.68		
Cusp of aortic valve.	27.4	11.6	4.48		
	27.4	11.6	4.50	15	2.01
	27.4	11.6	4.49		
Capsule of spleen.	23.8	10.4	4.63		
	23.3	10.5	4.41	17	1.86
	23.6	10.5	4.52		
Lung lymph node.	33.1	14.8	4.76		
	33.2	14.7	4.89	13	1.95
	33.2	14.8	4.83		
Mesenteric lymph node.	31.1	13.6	4.70		
	30.6	13.6	4.79	14	1.96
	30.9	13.6	4.75		
Fibroid Specimen A (chalk).	31.7	13.2	5.84		
	31.3	13.2	5.70	17	1.98
	31.5	13.2	5.77		

approached that of mature bone. His results have been confirmed by Herrmann (5).

These results, however, are not comparable with ours because of the different analytical procedures employed (6). Their values for P are those for total phosphorus and not for inorganic phos-

phorus, and their values of Ca are those for total calcium and not for residual calcium. The latter quantity cannot be calculated from their data since they reported no CO_2 analyses.

The abnormally high ratios of 2.23, 2.23, and 2.18 obtained in the case of calcified fibroids of the uterus take on added interest when compared with the high ratio of 2.23 ± 0.03 obtained for primary calcification. The possible significance of these high ratios is discussed in a subsequent paper (7).

TABLE II.

Calcified Fibroid of Uterus (Human).

Specimen.	Ca	P	CO_2	$\frac{\text{Carbonate Ca}}{\text{Total Ca}}$	$\frac{\text{Residual Ca}}{\text{P}}$
	per cent	*per cent*	*per cent*	*per cent*	
A (hard).	23.6	8.9	3.72		
	23.8	9.2	3.69	14	2.23
	23.7	9.1	3.71		
B	29.9	11.6	4.09		
	29.7	11.7	4.11	13	2.23
	29.8	11.7	4.10		
C	26.1	10.2	4.22		
	25.7	10.2	3.96	14	2.18
	25.9	10.2	4.09		

SUMMARY.

1. Seven specimens of pathologically calcified tissues were analyzed with the micro technique; all gave normal values for the ratio residual Ca : P.

2. Abnormal values for this ratio were obtained in analyses of calcified fibroids of the uterus.

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COMPOSITION OF BONE.

III. PHYSICOCHEMICAL MECHANISM.*

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INTRODUCTION.

A number of theories have been advanced to account for the deposition of bone salts. Freudenberg and György (1) consider that calcium first deposits as an organic calcium compound which then unites with phosphate, and that subsequently the calcium-organo-phosphate decomposes to give calcium phosphate. Dhar (2) attempts to explain it mainly from the point of view of colloidal phenomena. Robison (3) and his coworkers assign a prominent rôle in calcification to an enzyme in bone that converts organic phosphorus compounds into inorganic phosphates. Wells (4) and others have suggested that a local decrease in the CO_2 content or in the acidity of the tissue fluids may account for calcification.

The deposition of bone salts has also been attributed to simple precipitation of insoluble salts. Holt, La Mer, and Chown (5) in their notable investigation were the first to apply the modern theories of solution to this problem. More recently, Hastings, Murray, and Sendroy (6) studied the solubilities of calcium carbonate and tertiary calcium phosphate. These latter studies were also made from the point of view of the theory of complete dissociation of electrolytes, and constitute an important advance in that they contributed improved theoretical considerations.

From these last two investigations it appears evident that the ion product of $Ca_3(PO_4)_2$ is not the quantity which governs the deposition of calcium phosphate, and that the mechanism of calcification still remains obscure. This might be taken to mean that solubility product considerations are not applicable to this problem and that the solution must be sought elsewhere. However there is another alternative. It is possible that valuable

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information regarding calcification may yet be obtained with the aid of ion products if we employ the ion product of $CaHPO_4$ instead of that of $Ca_3(PO_4)_2$.

Knowledge of the composition of primary calcification, *i.e.* of the composition of the bone salts immediately after deposition, would probably throw considerable light on the mechanism involved in calcification. The attempts which have been made to elucidate the nature of the processes concerned in the deposition of bone salts have been made on the basis of analyses of old bone. There are a number of considerations which led us to suspect that the composition of primary calcification may be different from that of old bone. Just as precipitates of calcium phosphate change in composition when they remain in contact with the solutions from which they have been precipitated, so it seemed possible that the calcium salts after being deposited in the cartilage might subsequently undergo a change in composition to that obtained on analysis of old bone.

In the fourth paper of this series we give the results of analyses of primary calcification which were performed with our micro technique. In this paper we present the considerations which led us to suspect that $Ca_3(PO_4)_2$ is not deposited from serum and that new calcification may differ in composition from old bone. In outline there are: (1) the presence of $Ca_3(PO_4)_2$ in bone has never been demonstrated; (2) a chemical compound of the formula $Ca_3(PO_4)_2$ has never been prepared even from simple inorganic solutions; (3) the ion product $[Ca^{++}]^3 \times [PO_4{\equiv}]^2$ does not appear to be the quantity which determines calcification, either *in vivo* or *in vitro*; (4) when solutions of calcium and phosphate are mixed the initial precipitate is frequently $CaHPO_4$; this precipitate changes, on standing, to a solid phase whose empirical composition approximates $Ca_3(PO_4)_2$; (5) calcification is not obtained experimentally or clinically when the ion product $[Ca^{++}] \times [HPO_4{=}]$ is markedly below $K'_{s.p.}$ $CaHPO_4$;* calcification is obtained when the ion product for $CaHPO_4$ is close to $K'_{s.p.}$ $CaHPO_4$; (6) the experimental validity of the Ca × P product indicates that calcification is due to a second order reaction.

* The roman and italic letters for K in the forms $K_{s.p.}$ and $pK_{s.p.}$, *etc.*, are used interchangeably in this paper; there is no special significance attached to the italic form.—Publisher's note.

Precipitation of Calcium Phosphate.

The precipitation of calcium phosphate from both inorganic and biological systems has been investigated from the point of view of the ion product $[Ca^{++}]^3 \times [PO_4{\equiv}]^2$ and $K'_{s.p.}$ $Ca_3(PO_4)_2$. The results obtained show that these quantities are either inapplicable or inadequate. Holt, La Mer, and Chown postulated supersaturation with respect to $Ca_3(PO_4)_2$ in order to account for the peculiarities observed. Sendroy and Hastings concluded that the theory of supersaturation is inadequate. Klinke (7), Robison and Soames (3), and Greenwald (8) have presented additional evidence against the theory of supersaturation. Moreover, the experiments of Shipley, Kramer, and Howland (9), of Kramer, Shelling, and Orent (10), and of Shipley and Holt (11) have demonstrated that aqueous solutions of the same inorganic composition as serum may hold in solution, without the aid of organic compounds, the same amounts of calcium and phosphate as are found in serum.

In discussing the composition of the bone salts it is generally taken for granted that the calcium phosphate is present as $Ca_3(PO_4)_2$. We wish to emphasize that *the presence of $Ca_3(PO_4)_2$ in bone has yet to be demonstrated.* An examination of the literature (Dolhaine (1), Robison and Soames (3), and (12–34)) shows that the nature of the calcium phosphate compound in bone has never been elucidated. The belief that bone contains $Ca_3(PO_4)_2$ has gained universal acceptance apparently only on the basis of the empirical Ca : P ratio obtained from analyses. Moreover, when the analytical data are examined, it is seen that it has never been demonstrated that bone contains an empirical Ca : P ratio which is the same as that of $Ca_3(PO_4)_2$.

There are astonishingly few analyses of bone which have been made on unashed material by accurate analytical procedures. These very scanty data, as well as the numerous analyses of ashed material, show a Ca : P ratio only *approximating* that of $Ca_3(PO_4)_2$. The deviations from the theoretical ratio have been ascribed to errors in experimental technique. This may or may not be so, but it is striking that a similar situation exists as regards tricalcium phosphate prepared by precipitation from solution. This reaction is usually written

$$3Ca^{++} + 2PO_4^{\equiv} \rightleftharpoons \downarrow Ca_3(PO_4)_2$$

We have been unable to find any evidence of the existence of a molecular species with the formula $Ca_3(PO_4)_2$. Precipitates of this "substance" rarely have the correct empirical composition and they cannot be purified by recrystallization. The evidence is such as to lead us to suspect that there may be no such chemical entity as $Ca_3(PO_4)_2$. No one has succeeded in preparing it by precipitation (12, 35–48), bearing out the theoretical objections to such a reaction on the grounds that fifth order reactions do not occur (49). We do not take the position that there can be no compound with this formula. It may be found possible to synthesize it by other methods, but so far no one has succeeded in preparing it by precipitation.

As a result of the foregoing considerations we have sought for another explanation of the mechanism of calcification. Dicalcium phosphate is also a slightly soluble substance. Moreover it is a definite molecular entity with characteristic crystal forms. In addition, the formation of a precipitate of $CaHPO_4$ is due to a second order reaction; *i.e.*,

$$Ca^{++} + HPO_4^{=} \rightleftharpoons \downarrow CaHPO_4$$

It is generally agreed that the precipitate which is deposited from an acid solution is crystalline $CaHPO_4$. The confusion exists only as regards the nature of the substance deposited from neutral or alkaline solutions. It is also generally agreed that when the precipitated $CaHPO_4$ is allowed to stand in contact with the supernatant solution, a subsequent reaction occurs—the solution becomes more acid and the solid phase becomes more basic. The confusion here is concerning the composition of the solid phase at the final equilibrium.

The titration curves of phosphoric acid obtained by various investigators (43, 50–52) constitute additional strong evidence for the precipitation of $CaHPO_4$. Since the pH remains constant at about 7 during the addition of the 2nd and 3rd equivalents of $Ca(OH)_2$, it means that approximately half of the total phosphate is present as $H_2PO_4^-$. The reactions which occur throughout this range therefore appear to be

$$H_2PO_4^- + OH^- \rightleftharpoons H_2O + HPO_4^{=}$$

$$HPO_4^{=} + Ca^{++} \rightleftharpoons \downarrow CaHPO_4$$

$$Ca^{++} + 2OH^- \rightleftharpoons \downarrow Ca(OH)_2$$

Precipitates whose Ca : P ratio is greater than 1.29 are said to be mixtures, in varying proportions, of $CaHPO_4$ and $Ca(OH)_2$ (39, 40).

Solubility Product of $CaHPO_4$.

There have been a number of investigations dealing with the solubility of dicalcium phosphate, but the earlier work cannot be used in evaluating $K'_{s.p.}$ $CaHPO_4$ because the pH of the solutions was not determined. Behrendt (53), Kugelmass and Shohl (54), Holt, La Mer, and Chown (5), and Domontovitsch and Sarubina (55) have made determinations of $K'_{s.p.}$ $CaHPO_4$ taking the pH of the solutions into consideration.

We have recalculated $K'_{s.p.}CaHPO_4$ from the data of the last three investigations mentioned above, using the expression

$$K'_{s.p.}\ CaHPO_4 = [Ca^{++}] \times [HPO_4^{=}] \qquad (1)$$

We have set $[Ca^{++}]$ equal to the total calcium concentration as obtained by analysis. The value of $[HPO_4^{=}]$ was obtained by means of the expression

$$[HPO_4^{=}] = \frac{[P]}{1 + 10^{(pK_2' - pH)}} \qquad (2)$$

in which [P] is the concentration of phosphate obtained by analysis, and pK_2' was calculated from the expression given by Sendroy and Hastings

$$pK_2' = 7.15 - 1.25\sqrt{\mu} \qquad (3)$$

Equation (2) was obtained by rearrangement of equation (15) of Sendroy and Hastings ((6), p. 785).

When the values of $K'_{s.p.}CaHPO_4$ obtained in this way are plotted against $\sqrt{\mu}$, we obtain

$$pK'_{s.p.}\ CaHPO_4 = 6.4 - 2.3\sqrt{\mu} \text{ for } 38° \qquad (3,a)$$

This linear expression describes the data from zero ionic strength up to values of 0.38 for $\sqrt{\mu}$; *i.e.*, up to approximately the ionic strength of serum. For the range between room temperature and body temperature, up to $\sqrt{\mu} = 0.38$,

$$pK'_{s.p.}\ CaHPO_4 = 6.5 - 2.6\sqrt{\mu} \text{ for } 19\text{–}38°$$

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These values are probably correct to ± 0.1. At zero ionic strength, therefore,

$$pK_{s.p.}\ CaHPO_4 = 6.4 \pm 0.1 \text{ at } 38°$$

i.e.,

$$K_{s.p.}\ CaHPO_4 = (4 \pm 1) \times 10^{-7}$$

The value $2.3\sqrt{\mu}$ for $\Delta\ pK'_{s.p.}$ differs somewhat from the value $4\sqrt{\mu}$ given by Holt, La Mer, and Chown. Our value for $K_{s.p.}$ $CaHPO_4$ at zero ionic strength agrees well with their value; we have, moreover, estimated the probable accuracy of this value.

Subsequent Reaction and Final Equilibrium.

When solutions containing calcium are mixed with solutions containing phosphate so that the resulting solution is acid, it is generally agreed that the precipitate which deposits from solution is secondary calcium phosphate. It is also generally agreed that this precipitate subsequently reacts with the supernatant solution; the solution becomes more acid and the solid phase more basic; *i.e.*, the ratio Ca: P in the solid phase becomes larger.

It has also been demonstrated by Rindell (56) and by Buch (57) that when crystals of secondary phosphate are equilibrated with water the composition of the solid phase changes. The nature and mechanism of these reactions are still unknown. The reaction has been represented, for example, as follows:

$$\underset{\text{(solid)}}{CaHPO_4} + H_2O \longrightarrow \underset{\text{(solid)}}{Ca_3(PO_4)_2} + \text{acid solution}$$

The formula $Ca_3(PO_4)_2$ appears frequently in such equations because the solid phase at equilibrium frequently has a Ca: P ratio approximating 1.94, the theoretical value for a compound with the formula $Ca_3(PO_4)_2$.

The conditions which determine the final equilibrium are unknown. Holt, La Mer, and Chown attempted to explain this behavior using $K'_{s.p.}Ca_3(PO_4)_2$ as the criterion of the final equilibrium, postulating prolonged supersaturation with respect to $Ca_3(PO_4)_2$. This theory, however, does not account satisfactorily for the experimental results.

TABLE I.

Solutions in Equilibrium with Precipitates at 38°.

Experiment No.*	$\sqrt{\mu}$	Ca	pH	Ca:P in solid phase.	$\log \frac{[Ca^{++}]}{[H^+]}$.
		mols × 10^3			
†	0.025	0.20	6.40	2.06	2.7
CC 6	0.063	1.37	5.31	1.93	2.5
Z 1	0.064	1.46	5.30	1.94	2.5
Z 7	0.074	1.19	5.42	1.94	2.5
DD 1	0.075	1.65	5.15	1.94	2.4
CC 5	0.075	2.03	5.18	1.94	2.5
V 1	0.076	1.95	5.21	1.94	2.5
CC 4	0.084	2.74	4.96	1.96	2.4
Z 8	0.084	1.01	5.70	1.94	2.7
Z 2	0.085	1.48	5.46	1.94	2.6
‡	0.085	2.50	5.06	1.94	2.5
DD 2	0.091	1.98	5.44	1.94	2.7
V 2	0.099	1.98	5.27	1.94	2.6
CC 3	0.101	3.44	5.00	1.91	2.5
M 9	0.103	3.50	4.87	1.94	2.4
DD 3	0.103	2.20	5.51	1.94	2.9
M 8	0.109	4.00	4.84	1.94	2.4
M 7	0.112	4.15	4.86	1.94	2.5
CC 2	0.112	4.15	5.04	1.94	2.7
M 6	0.120	4.55	4.94	1.94	2.6
CC 1	0.122	5.02	5.04	1.96	2.7
M 5	0.123	5.00	5.03	1.94	2.7
Z 3	0.127	1.56	5.55	1.94	2.7
S 2	0.13	5.70	5.08	1.26	2.8
M 4	0.13	5.30	5.06	1.29	2.8
V 3	0.163	1.99	5.33	1.94	2.6
Z 4	0.227	1.66	5.67	1.94	2.9
V 4	0.273	2.06	5.46	1.94	2.8
U 9	0.34	5.74	5.39	1.29	3.2
U 10	0.34	5.45	5.36	1.29	3.1
V 5	0.363	2.10	5.51	1.94	2.8
V 6	0.363	2.20	5.52	1.94	2.9
AA 11	0.374	0.115	7.70	2.05–2.20	3.8
AA 10	0.374	0.153	7.10	2.05–2.20	3.3
AA 9	0.376	0.125	7.40	2.05–2.20	3.5
AA 5	0.376	0.145	6.92	2.05–2.20	3.1
AA 8	0.377	0.095	7.29	2.05–2.20	3.3
AA 7	0.378	0.133	7.22	2.05–2.20	3.3
AA 6	0.380	0.153	7.22	2.05–2.20	3.4
AA 2	0.381	0.125	6.95	2.05–2.20	3.1

* The data are taken from Holt, La Mer, and Chown (5).

† The seventh experiment of Holt, La Mer, and Chown ((5), Table I, p. 515).

‡ The experiment performed 9 days after mixing ((5), Table I, p. 569).

TABLE I—*Concluded.*

Experiment No.*	$\sqrt{\mu}$	Ca	pH	Ca:P in solid phase.	$\log \frac{[Ca^{++}]}{[H^+]}$.
		mols × 10^3			
AA 3	0.381	0.150	7.05	2.05–2.20	3.2
AA 4	0.381	0.123	7.05	2.05–2.20	3.1
AA 1	0.382	0.143	7.02	2.05–2.20	3.2
Z 5	0.445	2.00	5.94	1.94	3.2
T 7	0.509	3.07	5.53	1.94	3.0
Z 6	0.619	2.44	5.99	1.94	3.4
Y 4	0.65	8.31	6.28	1.29	4.2
Y 6	0.65	8.50	6.31	1.29	4.2

We have found an interesting regularity in all the experiments in which final equilibrium was attained by shaking precipitates of calcium phosphate with their supernatant solutions. When the analytically determined calcium concentration of the solution, expressed in mols per liter, is divided by the hydrogen ion concentration a constant is obtained. The results are shown in Table I. The last column shows that for a given ionic strength

$$\log \frac{[Ca^{++}]}{[H^+]\dagger} = \text{a constant} \tag{4}$$

therefore

$$\frac{[Ca^{++}]}{[H^+]} = K'_{\text{equil.}} \tag{5}$$

Moreover $\log K'_{\text{equil.}}$ increases with increasing ionic strength in accordance with modern theories of solution. When the data are given graphically, it is seen that there is a straight line relationship between $\log K'_{\text{equil.}}$ and $\sqrt{\mu}$ in which, at 38°,

$$\log K'_{\text{equil.}} = 2.4 + 2.2\sqrt{\mu} \tag{6}$$

i.e.,

$$\log K_{\text{equil.}} = 2.4 \text{ at zero ionic strength} \tag{7}$$

† We have followed the notation of Michaelis with regard to hydrogen ion, in which *"the symbol [H^+] will represent not the concentration but the activity of H-ions, and pH its negative logarithm."* (L. Michaelis, Hydrogen ion concentration, translated by W. A. Perlzweig, Baltimore, 1926, i, p. 128.) Hastings, Murray, and Sendroy used the activity symbols α_{H^+} and $p\alpha_{H^+}$.

or

$$K_{\text{equil.}} = 10^{2.4} \tag{8}$$

Furthermore this same ratio gives a similar constant at room temperature as can be seen from Table II. The ionic strength cannot be calculated for these solutions because the concentrations of the ions other than calcium, hydrogen, and phosphate are not given. The authors state however, that the "Gesamtkonzentration der Elektrolyte" ranged between 0.04 and 0.10 M.

TABLE II.

Solutions in Equilibrium with Precipitates at 19–22°.

Data of Domontovitsch and Sarubina ((55) p. 466).

Experiment No.	Ca	pH	$\log \frac{[Ca^{++}]}{[H^+]}$
	mols $\times 10^3$		
4	3.32	5.40	2.9
	1.30	5.86	3.0
	0.64	6.26	3.1
5	0.62	5.96	2.8
	1.60	5.52*	2.7
	1.40	5.54	2.7
	32.4	4.52	3.0
6	27.6	4.52	3.0
	25.7	4.64	3.1
	31.6	4.44	2.9
	26.2	4.48	2.9
	19.5	4.78	3.1
	30.0	4.42	2.9
	15.2	4.82	3.0
	14.7	4.87	3.0
	12.2	5.02	3.1

* The value 3.52 in the original is evidently a typographical error.

The $\sqrt{\mu}$ for these solutions, therefore, probably ranges between 0.15 and 0.30. At 38° log $K'_{\text{equil.}}$ varies from 2.7 to 3.1 in this range; at room temperature it varies also from 2.7 to 3.1. This indicates that the temperature coefficient of $K'_{\text{equil.}}$ is not very large, since there is little difference between the values obtained at room and body temperatures.

There are then forty-eight experiments at 38° and also sixteen

experiments at room temperature in which solutions containing calcium and phosphate were equilibrated with precipitates of calcium phosphate. The solid phase in some instances was secondary calcium phosphate, in others it approximated the composition of tricalcium phosphate, and in others it was more basic than tricalcium phosphate. *It is remarkable that the ratio* $[Ca^{++}] : [H^+]$ *is a constant regardless of the composition of the solid phase.*

We desire to emphasize that this equilibrium constant $K'_{\text{equil.}}$ is an empirical constant obtained directly from experimental data and that it involves no assumptions regarding the mechanism of the chemical reactions concerned. This experimental constancy may be written in two other ways. According to Sendroy and Hastings

$$\text{pH} = \text{pK}_2' + \log \frac{[\text{HPO}_4^{=}]}{[\text{H}_2\text{PO}_4^{-}]} \tag{9}$$

or

$$[\text{H}^+] = K_2' \frac{[\text{H}_2\text{PO}_4^{-}]}{[\text{HPO}_4^{=}]} \tag{10}$$

Substituting this value of $[\text{H}^+]$ in equation (5) we get

$$\frac{[\text{Ca}^{++}] \times [\text{HPO}_4^{=}]}{[\text{H}_2\text{PO}_4^{-}]} = K_2' \times K'_{\text{equil.}} \tag{11}$$

at zero ionic strength $K_2' = 10^{-7.15}$ and $K'_{\text{equil.}} = 10^{2.4}$. Substituting these values in equation (11) we get

$$\underline{K_{\text{equil.}}} = \frac{[\text{Ca}^{++}] \times [\text{HPO}_4^{=}]}{[\text{H}_2\text{PO}_4^{-}]} = 10^{-4.8} \tag{12}$$

From equation (12) it appears that the final equilibrium which is attained depends upon both the ion product of $CaHPO_4$ and hydrolysis of the $[HPO_4^{=}]$ ion to $[H_2PO_4^{-}]$. It is significant that this constant holds not only when the solid phase is $CaHPO_4$, but also when it is tricalcium phosphate and when it is more basic than tricalcium phosphate. This is in accord with the opinion

which we expressed in preceding sections that the precipitate throughout the entire range may contain $CaHPO_4$.

In alkaline solutions the Ca:P ratio in the precipitate which forms immediately after mixing is greater than 1.29; in acid solutions under some conditions the initial solid phase with a ratio of 1.29 changes to one which at equilibrium has a ratio approximating 1.94. In what form is this additional calcium? This question cannot be definitely answered at present. It is possible that the solid phase removes calcium from the solution by adsorption. It is also possible that there is a deposition in some way of some basic calcium compound like CaO, or $Ca(OH)_2$. It is interesting to note that the experimental statement

$$\frac{[Ca^{++}]}{[H^+]} = K'_{equil.} \tag{5}$$

may be written

$$[Ca^{++}] \times [OH^-] = K'_{equil.} \times 10^{-14} \tag{13}$$

since

$$[H^+] \times [OH^-] = k_w = 10^{-14} \tag{14}$$

On multiplying equations (13) and (14) we get

$$([Ca^{++}] \times [OH^-]^2) \times [H^+] = K'_{equil.} \times 10^{-28} = \underline{\underline{K'_{equil.}}} \tag{15}$$

At zero ionic strength

$$\underline{\underline{K_{equil.}}} = ([Ca^{++}] \times [OH^-]^2) \times [H^+] = 10^{-21.6} \tag{16}$$

From this calculation it is seen that when the experimentally obtained constant is multiplied by the square of the ion product of water, *i.e.* by 10^{-28}, another equilibrium constant is obtained. This constant also may have theoretical significance, for it is dependent upon the ion product of $Ca(OH)_2$ and the hydrogen ion concentration.

Calcification in Vitro.

Shipley, Kramer, and Howland (9) obtained calcification *in vitro* by using solutions containing only inorganic constituents.

We have repeatedly performed experiments on calcification *in vitro* and wish to point out that in such experiments *calcification* is obtained and not *precipitation;* calcium phosphate is deposited in a narrow zone across the epiphyses, exactly analogous to the line test obtained in calcification *in vivo.* It is usually deposited in no other part of the wide ricketic zone, nor is any precipitate formed in the supernatant solution. Calcification may therefore be studied in systems in which the composition of the solution may be varied at will.

Inorganic serum solutions were prepared and the pH was adjusted to 7.3; these solutions resemble serum closely except that

TABLE III.

Ion Product of $CaHPO_4$ in Inorganic Serum Solutions.

$\sqrt{\mu} = 0.38$, pH = 7.35, Ca = 10.0 mg. per 100 cc.

P per 100 cc.	Ca × P.	$[Ca^{++}] \times [HPO_4^{=}]$.	
mg.			
6.0	60	4.0×10^{-6}	Calcification.
5.0	50	3.3×10^{-6}	
4.0	40	2.7×10^{-6}	
3.5	**35**	$\mathbf{2.3 \times 10^{-6}}$	
3.0	30	2.0×10^{-6}	No calcification.
2.0	20	1.3×10^{-6}	
1.5	15	1.0×10^{-6}	

$K'_{s.p.}$ $CaHPO_4 = (3.2 \pm 0.8) \times 10^{-6}$ for $\sqrt{\mu} = 0.38$; *i.e.*, between 2.4×10^{-6} and 4.0×10^{-6}.

they contain no organic material. When these solutions were employed it was found that calcification was obtained when the product Ca × P exceeded 40; no calcification was obtained when this product was below 30.‡ We have calculated the ion product of $CaHPO_4$ in inorganic serum solutions having a constant calcium content of 10 mg. per cent and a phosphorus content ranging from 1.5 to 6.0 mg. per cent. The results are given in Table III.

When these ion products are compared with $K'_{s.p.}$ $CaHPO_4$ for that ionic strength, the striking observation is made that *these ion products are of the same order of magnitude as the solubility*

‡ This is the empirical product of Howland and Kramer (58) in which the concentrations of Ca and P are expressed in mg. per 100 cc.

product of $CaHPO_4$. When $\sqrt{\mu}$ is 0.38, $pK'_{s.p.}$ $CaHPO_4 = 5.5$,§ with an estimated error of $\pm$ 0.1; *i.e.*, $K'_{s.p.}CaHPO_4 = (3.2 \pm 0.8) \times 10^{-6}$. This means that for these solutions the solubility product lies somewhere between 2.4×10^{-6} and 4.0×10^{-6}, while the ion products range from 1.0×10^{-6} to 4.0×10^{-6}. *Solutions with a Ca $\times$ P product less than 30 are therefore undersaturated with respect to $CaHPO_4$.* This may account for the fact that calcification *in vitro* cannot be obtained in solutions with a Ca $\times$ P product less than 30. Solutions with Ca $\times$ P products from 35 to 60 have for the ion product $[Ca^{++}] \times [HPO_4^{=}]$ values which are very close to the solubility product of $CaHPO_4$.‖

Shipley, Kramer, and Howland have shown that, when the Ca $\times$ P product is 35, calcification is obtained *in vitro* not only when the calcium is 10 mg. and the phosphorus is 3.5 mg., but also when the calcium is 5 mg. and the phosphorus is 7 mg. The importance of this empirical Ca $\times$ P product was first pointed out by Howland and Kramer (58, 59). They showed that in young children and in young rats when the product of calcium and inorganic phosphorus in serum was below 30, active rickets was present; when the Ca $\times$ P product was above 40, rickets was either absent or healing had set in. The dividing value between calcification and absence of calcification was in the neighborhood of 35. They observed healing rickets in one instance when the calcium was 5.5 mg.; the Ca $\times$ P product in this case was 44. Thus the empirical Ca $\times$ P product applies in the same way to calcification *in vivo* and *in vitro*.

The fact that this empirical product has proved so valuable a guide clinically and experimentally gives rise to the thought that there may be a theoretical basis for it. This empirical product would have theoretical validity if the calcium were deposited as the result of a second order reaction such as

$$Ca^{++} + HPO_4^{=} \rightleftharpoons \downarrow CaHPO_4$$

§ These values for $pK'_{s.p.}$ $CaHPO_4$ are taken from the curve obtained when the values of $pK'_{s.p.}$ $CaHPO_4$ are plotted against $\sqrt{\mu}$. They may also be calculated from equation (3,a), p. 129.

‖ The curve of Holt, La Mer, and Chown ((5), Fig. 10) gives a value of 5.6 for $pK'_{s.p.}$ $CaHPO_4$ at this ionic strength; *i.e.*, $K'_{s.p.}$ $CaHPO_4 = 2.5 \times 10^{-6}$. This is the value obtained from their theoretical curve in which $\Delta pK_{s.p.} = 4\sqrt{\mu}$.

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because

$$Ca \times P \propto [Ca^{++}] \times [HPO_4^{=}]$$

This function is a linear one and is, at the pH of serum,

$$Ca \times P = 1.5 \times 10^7 ([Ca^{++}] \times [HPO_4^{=}])$$

Effect of pH.—The value of the product Ca × P is limited to the pH of serum; it does not hold in solutions whose reaction differs markedly from a pH of 7.3. Kramer, Shelling, and Orent (60) studied the effect of pH on calcification *in vitro* in this laboratory. Their results show that when the pH is below 7.0, calcification is not obtained even with a product of 50.

TABLE IV.

Inorganic Serum Solutions $\sqrt{\mu} = 0.38$.

Ca per 100 cc.	P per 100 cc.	pH	Ca × P.	$[Ca^{++}] \times [HPO_4^{=}]$.
mg.	mg.			
5.0	7.0	7.35	35	2.3×10^{-6}
7.8	6.5	7.35	51	3.4×10^{-6}
10.0	3.5	6.90	35	1.8×10^{-6}
10.0	3.5	6.80	35	1.7×10^{-6}
10.0	4.0	6.90	40	2.1×10^{-6}
10.0	4.0	6.80	40	1.9×10^{-6}
5.5	8.0	7.35	44	2.9×10^{-6}
5.5	8.0	6.80	44	2.1×10^{-6}

Although the empirical Ca × P product is not applicable in solutions more acid than serum, the theoretical ion product $[Ca^{++}] \times [HPO_4^{=}]$ still gives consistent results. These results are summarized in Table IV. It is seen from this table that empirical products from 35 to 44 have ion products of 2.3×10^{-6} or greater when the pH is 7.35; when the pH drops to slightly below 7.0 the ion products are less than 2.3×10^{-6}; *i.e.*, they are undersaturated with respect to $CaHPO_4$ even with an empirical Ca × P product of 44. It therefore appears that calcification *in vitro* is not obtained when the ion product $[Ca^{++}] \times [HPO_4^{=}]$ is less than 2.3×10^{-6}. This ion product has a wider applicability than the Ca × P product; it may therefore be found to be more

useful than the latter product. In that case, the approximate dividing value between calcification and no calcification may be taken as 2.5×10^{-6}.

Local Factors.—According to the above calculations these inorganic serum solutions at pH 7.3 are undersaturated with respect to $CaHPO_4$ when their Ca × P products are less than 30. For empirical products between 35 and 60 these solutions are probably very slightly undersaturated or just saturated with respect to $CaHPO_4$. This would account for the fact that these solutions are clear. Why then, when sections of ricketic bone are immersed in the latter solutions, should calcification occur? Furthermore, why should calcification take place in a definitely localized area of the sections?

The entire problem of the factors operating locally is still unsolved. It is obvious that these local factors are of importance else calcification would occur throughout the entire uncalcified zone. At the present time we can only venture a guess. It is possible that within the matrix¶ the pH is greater than 7.3 or that $\sqrt{\mu}$ may be less than 0.38. Either one, or both, of these factors may produce a local supersaturation and consequent precipitation of $CaHPO_4$.

If for any reason the inside of the matrix is more alkaline than the solution which bathes it, $[HPO_4^{=}]$ inside the matrix will be greater than in the solution even though the total [P] is the same, for $[HPO_4^{=}]$ will increase at the expense of $[H_2PO_4^{-}]$. This increase of $[HPO_4^{=}]$ may be just sufficient to cause $[Ca^{++}] \times [HPO_4^{=}]$ to exceed $K'_{s.p.}$ $CaHPO_4$ (see Robison and Soames (3)).

If for any reason the matrix fluid has an ionic strength lower than the outside solution, it will have a correspondingly lower value of $K'_{s.p.}$ $CaHPO_4$. Thus, for example, a value of 3.0×10^{-6} for the ion product $[Ca^{++}] \times [HPO_4^{=}]$ is less than that of the solubility product of $CaHPO_4$ in a solution with $\sqrt{\mu}$ equal to 0.38, since at that ionic strength $K'_{s.p.}$ $CaHPO_4$ has a value of 3.2×10^{-6}. The same value of 3.0×10^{-6} for the ion product $[Ca^{++}] \times [HPO_4^{=}]$ will be greater than $K'_{s.p.}$ $CaHPO_4$ in a similar solution

¶ According to Shipley, Kramer, and Howland (9), the calcium phosphate is deposited in the intercellular matrix, just as is the case when healing of rickets occurs *in vivo*.

differing from the first only in that $\sqrt{\mu}$ is 0.31 instead of 0.38, since at the lower ionic strength $K'_{s.p.}$ $CaHPO_4$ has a value of 2.0×10^{-6}.** A concentration of $[Ca^{++}]$ and $[HPO_4^{=}]$ which gives a slightly undersaturated solution in the outside fluid may therefore give a slightly supersaturated solution with consequent precipitation in the inside fluid.

Calcification in Vivo.

The chief difference between serum and inorganic serum solutions is that serum contains proteins and possibly other substances which "bind" calcium so that the equation $[Ca] = [Ca^{++}]$ does not hold for serum. This does not affect the validity of the considerations just presented. Inorganic serum solutions with $Ca \times P$ products less than 30 are, according to the above calculations, undersaturated with respect to $CaHPO_4$; serum with similar $Ca \times P$ products should therefore also be undersaturated with respect to $CaHPO_4$. The ion product $[Ca^{++}] \times [HPO_4^{=}]$ in serum with a given calcium and phosphorus content cannot be greater than the ion product in an inorganic serum solution with the same calcium and phosphorus content. If the value of the ion product is different it must be less in serum, the difference depending upon the amount of calcium bound in unionized form. If $CaHPO_4$ is the substance which deposits from solution, then, according to these calculations, serum is *undersaturated* with respect to calcium phosphate.

The possible rôle of $CaHPO_4$ was considered by Howland and Kramer (59). Although they believed that bone consisted of tricalcium phosphate, yet they realized that the ion product of $CaHPO_4$ might be important. They stated: "There must be a solubility product constant for Ca and HPO_4 ions which has a definite value at a given pH and temperature. When this constant is exceeded precipitation must take place. . . . While the ionic concentration cannot be measured we can prove that the general principle holds true by clinical observation using as factors for a rough product the total concentration of calcium and of phosphorus in the serum."

** See foot-note, §, p. 137.

It has not been demonstrated as yet that calcification occurs when the ion product $[Ca^{++}] \times [HPO_4^{=}]$ exceeds $K'_{s.p.}$ $CaHPO_4$. Our calculations show, however, that failure to calcify occurs when this ion product is definitely less than the solubility product of $CaHPO_4$.

That normal serum is very near the saturation point with respect to $CaHPO_4$ is also indicated by an experiment of Mond and Netter (61). These investigators shook serum with $CaHPO_4$ at body temperature. The serum contained 9.4 mg. per cent calcium at the beginning; at the end of an hour the serum calcium was still 9.4 mg. per cent.

CONCLUSION.

We have not discussed the precipitation of $CaCO_3$ nor the rôle of $K'_{s.p.}$ $CaCO_3$. It seems that calcium phosphate is the substance which determines calcification; when conditions are such that calcium phosphate is not deposited, then so far as our present knowledge goes, no bone salts are deposited. It is our present opinion that the rôle of $K'_{s.p.}$ $CaCO_3$ in calcification will not be made clear until the behavior of calcium phosphate is understood.

In conclusion we desire to differentiate sharply between (1) conclusions which we regard as definitely established and (2) theories which we have suggested to account for observed findings. The following résumé includes only those points which we consider as definitely established.

The presence in bones of a compound with the formula $Ca_3(PO_4)_2$ has never been demonstrated; there is no evidence which shows that bone contains calcium and phosphate in even the same empirical ratio as $Ca_3(PO_4)_2$. There are in the literature exceedingly few analyses of unashed bones accurate to 0.5 per cent; these few analyses indicate the presence of basic calcium in bone.

The existence of a substance with the formula $Ca_3(PO_4)_2$ has never been demonstrated, not even in simple inorganic systems. In spite of repeated attempts during the last 100 years no one has succeeded in preparing precipitates with even the empirical composition of $Ca_3(PO_4)_2$.

The ion product $[Ca^{++}]^3 \times [PO_4^{\equiv}]^2$ fails to account satisfactorily for either the formation of the initial precipitate from solution or for the final equilibrium between the solid phase and the super-

natant solution. It also fails to account satisfactorily for bone formation.

Secondary calcium phosphate is a definite chemical entity with well defined crystal forms. At 38° and at zero ionic strength the solubility product of $CaHPO_4$ is $(4 \pm 1) \times 10^{-7}$. Up to the ionic strength of serum the relation between the stoichiometric solubility product and the ionic strength is an agreement with modern theories of solution.

When final equilibrium is attained between a precipitate of calcium phosphate and its supernatant solution, following prolonged equilibration, it is found that $\frac{[Ca^{++}]}{[H^+]}$ is a constant. This empirical equilibrium constant varies with the ionic strength in agreement with modern theories of solution. It is noteworthy that this same constant is obtained regardless of the composition of the solid phase at equilibrium.

Calcification is not obtained *in vitro* when the ion product $[Ca^{++}] \times [HPO_4^{=}]$ in the external solution is definitely less than $K'_{s.p.}CaHPO_4$. Calcification *in vitro* is obtained when this ion product is very nearly equal to $K'_{s.p.}CaHPO_4$. At a pH of 7.3 this ion product is as applicable as the empirical Ca × P product; moreover it is applicable in more acid solutions where the empirical Ca × P product does not hold.

SUMMARY.

1. As a criterion for the formation of precipitates of calcium phosphates, the ion product $[Ca^{++}]^3 \times [PO_4^{\equiv}]^2$ is inadequate.

2. We have found no evidence which satisfactorily demonstrates the presence in bones of a compound with the formula $Ca_3(PO_4)_2$.

3. From data in the literature $pK_{s.p.}$ $CaHPO_4$ is calculated to be 6.4 ± 0.1 at 38°. At any ionic strength up to that of serum

$$pK'_{s.p.}\,CaHPO_4 = 6.4 - 2.3\sqrt{\mu}$$

4. The ion product $[Ca^{++}] \times [HPO_4^{=}]$ at which calcification begins, both *in vivo* and *in vitro*, is numerically equal to the value calculated for $K'_{s.p.}$ $CaHPO_4$ at the ionic strength of serum.

5. A theoretical basis is suggested for the empirical Ca × P product. In inorganic serum solutions with a pH of 7.35

$$Ca \times P = 1.5 \times 10^7 ([Ca^{++}] \times [HPO_4^{=}])$$

6. Inorganic serum solutions with empirical Ca × P products less than 30 appear to be *undersaturated* with respect to $CaHPO_4$; calcification is not obtained with these solutions.

7. The evidence indicates that ricketic serum is definitely undersaturated with respect to $CaHPO_4$ and that normal serum is slightly undersaturated with respect to this salt.

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COMPOSITION OF BONE.

IV. PRIMARY CALCIFICATION.*

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The chemistry of the calcium phosphates together with its bearing on calcification was discussed in the preceding communication (1). The evidence indicated that the composition of bone when first deposited might be different from the composition of old bone. In inorganic aqueous solutions the primary precipitate of $CaHPO_4$ with a Ca : P ratio of 1.29 undergoes transformation, in many cases, to a solid phase in which Ca : P approximates 1.94. Similarly, although in bone the ratio Ca : P also approximates 1.94, it seemed possible that this ratio might be different in freshly deposited bone.

This secondary reaction, which precipitates of calcium phosphate undergo, is completed in about 8 days in inorganic solutions as Holt, La Mer, and Chown (2) have shown. If a similar reaction occurs *in vivo* its detection would require that analysis be made on bone that is not more than a few days old. We have utilized for such analysis the fresh calcification deposited in ricketic rats whose rickets had just begun to heal. The analytical procedures employed were those described in the first paper of this series (3).

Normal Rat Bone.

Sixteen specimens of bone from normal rats were analyzed with the micro technique. Calcium, phosphate, and CO_2 were determined in duplicate on each specimen. The analytical results are given in Table I. It is seen from this table that the ratio residual

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XUM

TABLE I.

Normal rat.

Age.	Bones.	Ca	P	CO_3	$\frac{\text{Carbonate Ca}}{\text{Total Ca}} \times 100$.	$\frac{\text{Residual Ca}}{\text{P}}$.	Deviation.
days		*per cent*	*per cent*	*per cent*			
1	All.	13.7	6.4	1.23			
		13.2	6.5	1.32	9	1.89	−0.10
5	Leg.	15.8	7.2	1.62			
		15.9	7.5	1.61	9	1.95	−0.04
5	Rib.	11.5	5.4	1.24			
		12.1	5.4	1.31	10	1.96	−0.03
5	Head.	14.6	6.7	1.62			
		14.5	6.7	1.54	10	1.97	−0.02
13	Leg.	12.4	5.7	1.12			
		12.4	5.7	1.16	8	2.00	+0.01
15	"	11.4	5.6	1.36			
		12.3	5.7	1.22	10	1.88	−0.11
15	Rib.	11.6	5.3	1.14			
		11.6	5.2	1.03	9	2.00	+0.01
20	Leg.	13.4	6.4	1.30			
		13.2	6.2	1.28	9	1.92	−0.07
20	Rib.	11.5	5.1	1.06			
		11.7	5.1	1.09	8	2.08	+0.09
38	Leg.	13.9	5.8	1.61			
		13.9	5.9	1.54	10	2.12	+0.13
38	Rib.	13.2	6.1	1.18			
		13.0	5.9	1.20	8	2.00	+0.01
46	Leg.	18.8	7.8	2.57			
		18.6	7.8	2.49	12	2.10	+0.11
63	Shafts.	21.8	9.3	3.20			
		21.7	9.2	3.19	13	2.03	+0.04
5 mos.	Leg.	21.0	8.9	3.62			
		21.7	8.8	3.99	16	2.01	+0.02
Adult 1.	"	24.1	10.3	4.05			
		23.9	10.2	4.16	16	1.97	−0.02
" 2.	"	23.5	10.1	3.79			
		23.4	10.2	3.86	15	1.96	−0.03
						Mean = 1.99	*a.d.* = ±0.05 *A.D.* = ±0.01

Residual Ca: P = 1.99 ± 0.01.

Ca : P obtained with the micro technique has a mean value of 1.99 for normal rat bone. The average deviation from the mean, *a.d.*, is ±0.05, and *A.D.* = ±0.01. The mean value of 1.99 ±0.01 obtained with the micro technique is in agreement with similar results obtained by others using macro methods.

The values in the column entitled $\frac{\text{"carbonate Ca}}{\text{total Ca}} \times 100$" were obtained by means of the expression

$$\frac{\text{Per cent } CO_2 \times 91}{\text{Per cent Ca}} = \frac{\text{carbonate Ca}}{\text{total Ca}} \times 100$$

The per cent of CO_2 multiplied by 0.91 gives the per cent of calcium assumed to be present as bound to carbonate in $CaCO_3$; *i.e.*, this gives the per cent of carbonate calcium. Dividing per cent of carbonate calcium by per cent of total calcium and multiplying by 100 gives the values in this column.

It is seen from this column that the bones of very young rats contain less $CaCO_3$ in proportion to calcium phosphate than do bones of older rats. In young rats carbonate Ca constitutes only 8 to 10 per cent of the total Ca, while in adult rats it constitutes about 15 per cent. This means that in the bones of rats the proportion of $CaCO_3$ to the other calcium salts increases markedly with age. Transition values were obtained in rats 6 and 9 weeks old.

This difference in the carbonate content of the bones of young and old rats is not due to experimental error. If the CO_2 values in young rats were too low, or if the values of total Ca were too high, then these errors would be reflected in the ratio residual Ca : P. All the bones, from rats varying in age from 1 day to maturity, gave the same value within the experimental deviation for the ratio residual Ca : P; the change in carbonate content with increasing age is, therefore, a real one.

The analyses and ratios given in Table I are selected values. Some of these bones were analyzed in duplicate and some in triplicate; in one case five analyses were made of the same bone. All of these analyses were performed as described previously—the calcium, phosphorus, and CO_2 in each case were determined at least in duplicate. The ratios obtained in all of these analyses

XUM

TABLE II.

Normal Rat.

Summary of all ratios.

Age.	Bones.	Ratio.	Deviation.
days			
1 and 2	All.	1.89	−0.12
5	Leg.	1.95	−0.06
5	Rib.	1.96	−0.05
5	Head.	1.97	−0.04
		1.97	−0.04
		1.90	−0.11
13	Leg.	2.10	+0.09
		2.00	−0.01
15	"	1.88	−0.13
15	Rib.	2.12	+0.11
		2.00	−0.01
		2.02	+0.01
20	Leg.	1.94	−0.07
		2.05	+0.04
		1.92	−0.09
20	Rib.	2.08	+0.07
38	Leg.	1.93	−0.08
		2.12	+0.11
		2.12	+0.11
38	Rib.	2.21	+0.20
		2.02	+0.01
		2.00	−0.01
46	Leg.	2.14	+0.13
		2.10	+0.09
63	Shafts.	2.03	+0.02
5 mos.	Leg.	2.01	0.00
Adult 1.	"	1.98	−0.03
		1.99	−0.02
		2.00	−0.01
		1.97	−0.04
		2.04	+0.03
" 2.	"	2.04	+0.03
		2.03	+0.02
		1.96	−0.05
		1.98	−0.03
		Mean = 2.01	*a.d.* = ±0.06
			A.D. = ±0.01

Residual Ca: P = 2.01 ± 0.01.

are given in Table II. These ratios have all been given here and have all been included in calculating the mean and the *A.D.*, for the sake of comparison with the ratios given in Table VI. The value 2.21 for example is obviously erroneous (two subsequent analyses of the same bone gave ratios of 2.02 and 2.00); it has nevertheless been included. The mean obtained from *all* the analyses of normal rat bone is 2.01 ±0.01.

TABLE III.

Primary Calcification (Rat).

Healing induced with cod liver oil concentrate.

Bone.	No. of samples analyzed.	Ca	P	CO_2	$\frac{\text{Carbonate Ca}}{\text{Total Ca}} \times 100$	$\frac{\text{Residual Ca}}{\text{P}}$
		per cent	*per cent*	*per cent*		
Fresh calcification (lines).	4	7.8	3.1			
		7.5	3.0			
		7.9	2.9			
		7.6	2.9			
		7.7	2.8	0.89		
			2.9	0.93	11	2.38
			2.9	0.91		
Epiphyses and diaphyses.	3	10.1				
		9.9	4.2	1.46		
		10.2	4.1	1.43	13	2.10
		10.1	4.2	1.45		
Old bone (shafts).	1	15.6	6.4	2.78		
		15.5	6.3	2.61	16	2.05
		15.6	6.4	2.70		

Primary Calcification.

Rickets was produced in rats by feeding Steenbock's Ration 2965.[1] Severe rickets was obtained in 4 weeks; sections from the upper end of the tibia showed a wide layer of uncalcified cartilage and a wide metaphysis. Twenty-five of these rats were fed cod liver oil concentrate for 8 days. Autopsy showed that the rickets had begun to heal; there was a line of fresh calcification in the provisional zone of cartilage.

[1] For the composition of Ration 2965 see Steenbock, H., and Black, A., *J. Biol. Chem.*, 1925, lxiv, 263.

All the rats were killed, and the upper end of each tibia was removed and sectioned. Each section contained the center of ossification, the epiphyseal cartilage, the line of fresh calcification, the metaphysis, and a little of the shaft. Each section was cut in three places at right angles to the axis of the shaft; the different portions of the sections were combined in three groups and labelled "shafts," "epiphyses and diaphyses," and "lines." Each of these three portions of the upper end of the tibia was then analyzed by means of the micro technique. The results are given in Table III.

In each case calcium, phosphate, and CO_2 were determined at least in duplicate. In the case of the new bone from the lines there were four analyses for calcium and six analyses for phosphate. Separate samples were digested for the additional analyses; they do not represent merely aliquots of the same solution. It is seen that the shafts gave a ratio of 2.05; this is within the normal range. The epiphyses and diaphyses gave a ratio of 2.10; this is somewhat high, probably because there was some fresh calcification present in addition to the older bone. The fresh calcification of the lines gave a decidedly high ratio of 2.38.

A similar study was carried out on another set of ricketic rats except that irradiated yeast was employed as the antiricketic agent. In these rats the healing of the rickets was not confined to the test line, as there was also considerable fresh calcification in the metaphysis itself. The sections of the tibia were therefore divided into three groups somewhat different from those in the preceding set of rats; these groups were "lines," "metaphyses," and "shafts." The results are given in Table IV.

Table IV shows that the old bone from the shafts gave a ratio of 2.05; this is within the normal range. The metaphyses gave a high ratio of 2.25 and the lines gave a moderately high ratio of 2.16. In analyzing a normal bone it sometimes happens that two errors occur in which a high calcium value and a low phosphorus value are obtained simultaneously. This would result in a high Ca : P ratio. To rule out this possibility ten analyses were made for calcium and twelve for phosphorus. A number of samples of the material were digested and analyzed separately; the value 2.16 is calculated from the mean values so obtained.

A third study was made in which the healing of the rickets was induced by butter. To the ricketic diet was added 1 per cent but-

ter; this mixture was fed for 4 weeks. At the end of this period autopsy showed that there was fresh calcification in the metaphysis but not in the provisional zone. The metaphyses were removed as described above, and analyzed. The shafts were also analyzed. The results are given in Table V.

TABLE IV.

Primary Calcification (Rat).

Healing induced with irradiated yeast.

Bone.	Ca	P	CO_2	$\frac{\text{Carbonate Ca}}{\text{Total Ca}} \times 100.$	$\frac{\text{Residual Ca}}{\text{P}}$.
	per cent	*per cent*	*per cent*		
Fresh calcification (lines).	12.8	4.9			
	12.3	4.9			
	12.3	5.4			
	12.2	5.2			
	12.2	5.3			
	12.6	5.2			
	12.4	5.2			
	12.3	5.2			
	12.4	5.1			
	12.6	5.1			
	12.4	5.1	1.67		
		5.1	1.51	12	2.16
		5.1	1.59		
Fresh calcification (metaphyses).	11.7				
	12.2	4.6			
	11.9	4.6			
	12.2	4.8	1.64		
	12.1	4.9	1.48	12	2.25
	12.0	4.7	1.56		
Old bone (shafts).	20.4	8.5			
	20.7	8.5			
	20.7	8.5	3.43		
	20.8	8.7	3.41	15	2.05
	20.7	8.6	3.42		

The old bone in the shafts gave a normal ratio of 1.94; the new bone in the metaphyses gave a high ratio of 2.14. To exclude the possibility of this high ratio being due to experimental error, repeated analyses were made for calcium and phosphorus. The ratio in the last column was calculated from the mean values.

In another group of ricketic rats healing was induced by irradiated cholesterol. The deposition of fresh calcification occurred in the provisional zone of cartilage; the lines were removed and analyzed. The results are given in Table VI. It is seen that this gave a high Ca : P ratio of 2.24. For comparison the leg shafts of a number of normal rats of the same age, *i.e.* 63 days old, were removed and analyzed. A normal ratio of 2.03 was obtained.

TABLE V.

Primary Calcification (Rat).

Healing induced with butter.

Bone.	Ca	P	CO_2	$\frac{\text{Carbonate Ca}}{\text{Total Ca}} \times 100$.	$\frac{\text{Residual Ca}}{\text{P}}$.
	per cent	*per cent*	*per cent*		
Fresh calcification (metaphyses).	10.0	4.4			
	10.1	4.2			
	10.4	4.2			
	10.1	4.2			
	10.2	4.2			
	10.2	4.6			
	10.2	4.5			
		4.4			
		4.4			
		4.3	1.10		
		4.3	1.11	10	2.14
		4.3	1.11		
Old bone (shafts).	20.3	8.9			
	19.8	8.5			
	20.1	9.0	3.32		
		8.7	3.26	15	1.94
		8.8	3.29		

DISCUSSION.

In interpreting the results of analyses of bones it is customary to assume that all the CO_2 is present as $CaCO_3$ and that all the Mg is present as $Mg_3(PO_4)_2$. The carbonate calcium is deducted from the total calcium, and the phosphate equivalent to the magnesium is deducted from the total phosphate. It has been repeatedly found that the residual calcium and residual phosphate so obtained give a ratio of Ca : P which approximates that calcu-

lated from the formula $Ca_3(PO_4)_2$. When analyzed with our micro technique, normal rat bone gives a ratio for residual Ca : P which approximates 1.94.[2] The Ca : P ratios which we have obtained for normal rat bone by means of the micro technique are therefore in agreement with the results of previous investigators.

Proportion of $CaCO_3$.—Our analyses show that the proportion of $CaCO_3$ to calcium phosphate in bone increases with the age of

TABLE VI.

Primary Calcification (Rat).

Healing induced with irradiated cholesterol.

Bone.	Ca	P	CO_2	$\frac{\text{Carbonate Ca}}{\text{Total Ca}} \times 100$.	$\frac{\text{Residual Ca}}{\text{P}}$.
	per cent	*per cent*	*per cent*		
Fresh calcification (lines).	9.9	3.8			
	9.9	3.8			
	9.3	3.6			
	9.0	3.6			
	9.0	3.6			
	9.2	3.9			
	9.1	3.8			
	9.2	3.8			
	9.2	3.8			
	9.3	3.7			
		3.6	1.12		
		3.8	1.16	11	2.24
		3.7	1.14		
Old bone (shafts of normal rats 63 days old).		9.3			
	21.8	9.3	3.20		
	21.7	9.2	3.19	13	2.03
	21.8	9.3	3.20		

the animal. There are no recent analyses of unashed bones with which we may compare these results.

This question has apparently not been studied within the last 35 years; we have found no recent analyses, either of ashed or of unashed bone, which have bearing on this point. In 1855 Frémy (4) concluded that in humans there is, with increasing age, a relative increase of $CaCO_3$ with respect to

[2] The effect of the omission of the magnesium analysis is discussed in the first paper (3).

calcium phosphate. Von Recklinghausen (5) in 1858 concluded from his own analyses and from the work of Bibra (1844), of Stark (1845), of Heintz (1849), and of Frémy, that there was no observable difference in composition between the bones of young and those of old individuals. In 1860 Milne Edwards (6) reported that his own analyses agreed with those of Bibra and of Frémy in showing that the proportion of carbonate in the bones of young animals was smaller than in adult bone. Zalesky (7) in 1866 reviewed the literature and concluded that the bones of children of various ages have the same composition as those of adults, not only as a whole, but also as regards the proportion of individual constituents. He pointed out that the methods of determining CO_2 employed by previous investigators were defective. In 1872 Wildt (8) analyzed[3] the ashed bones of rabbits of different ages and found an increase in the proportion of CO_2 with increasing age.

Weiske (10) in 1889 analyzed the ashed bones of birds and found an increase in CaO and CO_2 with advancing age, while the P_2O_5 remained constant. Graffenberger (11) in 1891 analyzed the ashed bones of rabbits and also found an increase of the ratio calcium carbonate: calcium phosphate with increasing age.

The only analyses of unashed bone performed during the last 30 years with which we are acquainted are the few analyses of Gassmann (12) and those of Howland, Marriott, and Kramer (13). The results of the latter investigators show that the ratio of calcium phosphate to calcium carbonate is not necessarily constant; this proportion is different in ricketic and normal bones, and is different in normal human bone as compared with normal rat bone. The effect of age on this ratio was not studied.

Our findings are in agreement with those of Howland, Marriott, and Kramer as regards the increase in the proportion of $CaCO_3$ in rickets. From our tables it is seen that for normal rats approximately 2 months old, carbonate Ca:total Ca is about 13 per cent; for ricketic rats of the same age it is 15 and 16 per cent, the same as in normal adult rats.

Since the proportions of the inorganic constituents vary also with age, as we have just demonstrated, it is incorrect to assign a definite formula to bone. The use of the famous formulas $3Ca_3(PO_4)_2 \cdot CaCO_3$ of Hoppe-Seyler (14) and $\left[Ca\begin{pmatrix} OPO_3Ca \\ >Ca \\ OPO_3Ca \end{pmatrix}_3 \right] CO_3$ of Werner (15) should therefore be discontinued as incorrect and misleading.[4]

[3] The data of Wildt and of Weiske are also given by Forbes and Keith (9).

[4] De Jong has attempted to determine the nature of the "compound"

Primary Calcification.—The results of our analyses of fresh calcification are summarized in Table VII. It is seen that in freshly deposited bone salts the proportion of $CaCO_3$ is low, carbonate Ca : total Ca being about 11 per cent, while in the older bones of these same animals carbonate Ca : total Ca has a value of 15 to 16 per cent. This shows the same phenomenon as do the data in Table I; *i.e.*, that new bone contains a smaller proportion of $CaCO_3$ to total calcium than does older bone.

TABLE VII.

Primary Calcification (Rat).

Specimen.	Antiricketic factor.	$\frac{\text{Carbonate Ca}}{\text{Total Ca}} \times 100$.	$\frac{\text{Residual Ca}}{\text{P}}$.	Deviation.
Test lines.	Cod liver oil concentrate.	11	2.38	+0.15
Metaphyses.	Butter.	10	2.14	−0.09
Test lines.	Irradiated cholesterol.	11	2.24	+0.01
" "	Irradiated yeast.	12	2.16	−0.07
Metaphyses.	" "	12	2.25	+0.02
		11	Mean = 2.23	*a.d.* = ±0.07 *A.D.* = ±0.03

Primary calcification, residual Ca: P = 2.23 ± 0.03.
Old bone " " " = 1.99 ± 0.01.

The ratio residual Ca : P obtained for primary calcification is 2.23 ±0.03; for older bones analyzed with the same technique this ratio has the value 1.99 ±0.01. At the present time there

in bone by means of x-ray analysis on the basis that "The ratio $\frac{CaCO_3}{Ca_3(PO_4)_2}$ being found about the same everywhere it may readily be suspected that bones are built up chiefly by one compound." He employs the formula $3Ca_3(PO_4)_2 \cdot CaX_2$ (*Proc. Acad. Sc. Amsterdam*, 1926, xxix, 870).

appear to be several ways of interpreting this high Ca : P ratio in primary calcification. They are: (1) experimental error, (2) presence of an acid-soluble organic calcium compound, (3) presence of inorganic calcium in cartilage, and (4) presence of an insoluble basic calcium salt.

The results do not appear to be ascribable to experimental error. Five different specimens were analyzed. The analyses were made repeatedly, on separate samples of each specimen, thus ruling out the simultaneous occurrence of an accidental high calcium value and of an accidental low phosphorus value. In the second place, the ratios for fresh calcification are all high; if the deviations were due to experimental error, some ratios should be below normal. In the third place, the occurrence of ratios as high as 2.20 is quite rare when due to experimental error; only one such ratio was obtained in forty-four analyses, as is seen from Table VI of Paper I of this series. Primary calcification gave a ratio of 2.23 $\pm$0.03 as compared with 1.99 $\pm$0.01 for normal rat bone. When all the ratios obtained in analyses of normal rat bone were averaged, including even those shown by subsequent analyses to be too high, a mean value of 2.01 $\pm$0.01 was obtained. When 2.23 $\pm$0.03 is compared with 2.01 $\pm$ 0.01 it is seen that the accidental occurrence of high ratios does not account for the value obtained for primary calcification.

It is possible that the material analyzed for primary calcification contains an acid-soluble organic calcium compound. However, until the presence of such a compound is demonstrated it is simpler to assume that the apparent excess of calcium is present as inorganic calcium.

The cartilage itself may contain some inorganic calcium aside from the calcium due to the presence of bone salts. Thus even if the ratio of Ca : P in freshly deposited bone salts has a normal value of 1.94, the presence of additional inorganic calcium in the cartilage itself would increase the value of the ratio. At the present time, however, the simplest interpretation of the experimental findings is that the high Ca : P ratio is due to the presence of basic calcium in the freshly deposited bone salts.

From the considerations discussed in the preceding communication we had expected that primary calcification would consist of $CaHPO_4$, and would be found to give *low* ratios. Analysis,

however, gave *high* ratios. On reconsidering the chemistry of the calcium phosphates we noted that $CaHPO_4$ is obtained as a precipitate only from acid solution. Cameron and Seidell (16) found that the precipitate was $CaHPO_4$ when the supernatant solution was acid, and that when the solution was alkaline the precipitate always contained a larger proportion of lime than is required for $Ca_3(PO_4)_2$. Cameron and Bell (17) advanced the view that the solid phases were solid solutions whose composition varied from that of $CaHPO_4$ to that of pure $Ca(OH)_2$ and that the solid phase which is usually given as $Ca_3(PO_4)_2$ may be a mixture of $CaHPO_4$ and $Ca(OH)_2$. They suggested that there might be "only one series extending over the whole range in which the solids are dicalcium phosphate and lime."

Serum has an alkaline reaction; the tissue fluid from which the bone salts are deposited may likewise be alkaline. If this is the case, then a simultaneous deposition of $CaHPO_4$ and basic calcium might be expected on the basis of analogy to inorganic systems. This would account for the apparent significance of the ion product $[Ca^{++}] \times [HPO_4^{=}]$ in clinical and experimental calcification as discussed in the preceding paper, and also for the presence of high Ca : P ratios in primary calcification.

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SUMMARY.

1. When analyzed with the micro technique, unashed normal rat bone gave a value of 1.99 ±0.01 for the ratio residual Ca:P; this is in agreement with the results of analyses of large amounts of bone by macro methods.

2. The proportion of carbonate in normal rat bone increases with age. The ratio carbonate Ca : total Ca is about 8 to 10 per

cent in the bones of young rats and 15 to 16 per cent in those of adult rats.

3. The proportion of carbonate in ricketic rat bone is greater than in normal rat bone of the same age.

4. The proportion of carbonate in primary calcification is less than in the older bones of the same animals.

5. The ratio residual Ca : P in primary calcification has a high value of 2.23 ±0.03. This high ratio is interpreted as indicating the presence of a basic calcium salt in freshly deposited bone salts.

6. The composition of the primary calcification appears to be independent of the antiricketic reagent; high Ca : P ratios were obtained regardless of whether the fresh calcification was induced by cod liver oil concentrate, butter, irradiated cholesterol, or irradiated yeast.

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COMPOSITION OF BONE.

V. SOME PROPERTIES OF CALCIUM CITRATE.*

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The deposition of bone salts appears to be dependent upon physicochemical equilibria existing in serum. Calcium ion plays an important part in these equilibria and it is important to know what proportion of the total serum calcium is present as calcium ion. It is generally agreed that a large part of the serum calcium is bound in non-diffusible compounds by proteins. Moreover the opinion has been advanced by Greenwald (1) and supported by Klinke (2) that an additional part of the total calcium is present in serum in the form of a slightly ionized, diffusible organic calcium compound resembling calcium citrate. Sendroy and Hastings (3) also believe that there is strong evidence for the existence of a slightly ionized, diffusible calcium compound in serum. They suggested that this substance may be an organic compound or some slightly dissociable phosphate compound and pointed out the similarity between the parathyroid hormone and citrate solutions as regards their apparent ability to hold calcium in solution. The ultrafiltration experiments of Shelling and Maslow (4) also point to the formation of an unionized ultrafiltrable calcium citrate compound. These authors found that sodium citrate, when injected into the living animal or added to serum *in vitro*, increased markedly the per cent of ultrafiltrable calcium.

The rôle of citrate, and of substances that resemble citrate in their behavior towards calcium ions, may be an important one in calcification. Thus Shipley, Kramer, and Howland (5) found

* These experiments were reported before the Society for Experimental Biology and Medicine, March 16, 1927 (*Proc. Soc. Exp. Biol. and Med.*, 1927, xxiv, 624.)

that citrate inhibited calcification *in vitro* while lactate and acetate did not. Hastings, Murray, and Sendroy (6) studied the solubility of $CaCO_3$ in solutions of sodium citrate and concluded that the evidence seemed to indicate the formation of a slightly ionized calcium citrate compound. They also concluded that the parathyroid hormone seems to act towards calcium like citric acid. Although there is a considerable amount of indirect evidence which points to the binding of calcium by citrate to form a diffusible, unionized or slightly ionized substance, there is so far as we are aware no direct evidence. In this paper we are presenting conductivity measurements which give direct evidence for the binding of calcium by citrate.

Solubility of Calcium Citrate.

Our plan had been to make a concentrated solution of calcium citrate, and to follow the change in ionization with dilution by means of conductance measurements. This plan was abandoned because of peculiarities encountered in dissolving calcium citrate in water.

The solubility of $Ca_3(C_6H_5O_7)_2 \cdot 4H_2O$ is given (7) as 0.08496 gm. per 100 gm. of water at 18° and 0.0959 gm. per 100 gm. of water at 25°. We first attempted to dissolve 0.048 gm. of commercial calcium citrate[1] in 100 gm. of water, but the material did not dissolve completely; a considerable portion remained undissolved. An equal volume of water was added, but still solution was incomplete. We then took a smaller amount of the material, using first 0.024 gm. and then 0.011 gm. of calcium citrate per 100 cc. of water. The latter is about one-ninth the amount given as the solubility at 25°, but it did not dissolve, even on boiling. Protracted boiling for several hours finally caused all of the solid material to enter the liquid phase. Another sample, after standing on a hot plate for several days, also finally dissolved completely.

We then attempted to prepare calcium citrate by precipitation and encountered interesting colloidal phenomena. It is peculiar that a salt, which is said to increase in solubility with rise in temperature, is precipitated from solution by boiling. Thus Peterson, Wilson, McCoy, and Fred (8) prepared calcium citrate

[1] Two different specimens of calcium citrate, purchased from different sources, were used in these solubility experiments.

by mixing solutions of citric acid and lime water, "filtering and boiling for a few minutes to precipitate the calcium citrate."

Our experiences indicated that the preparation of solid calcium citrate may involve colloidal coagulation instead of simple precipitation, and that solution of the solid material may involve peptization rather than simple solution. There is another possible explanation for the fact that prolonged boiling caused the calcium citrate to dissolve; it may be that hydrolysis of the dissolved calcium citrate occurs, and that this hydrolysis results in solution of additional salt from the solid phase.

We did not investigate these peculiarities further since our primary purpose was to study the behavior of calcium ions in the presence of citrate ions in solution and not the equilibria existing between solutions of calcium citrate and the solid salt.[2] We therefore decided to investigate the behavior of the ions by means of conductivity titrations.

Conductivity Titrations.

The procedure employed in these electrical conductivity titrations consists essentially of the determination of the resistance of each of two solutions, and of mixtures of these two solutions in varying proportions.

The measurements were made at 25.0° ±0.1° with a cylindrical glass vessel about 2 cm. in diameter and removable electrodes of the Ostwald type. A Wheatstone bridge arrangement was used, in which a Kohlrausch slide wire bridge and a resistance box were employed. The measuring instruments were made by Leeds and Northrup. Three solutions were prepared; 0.00125 N NaCl, 0.005 N sodium citrate, and 0.005 N $CaCl_2$.

The $CaCl_2$ solution was made by decomposing a calculated weight of $CaCO_3$ with a small volume of HCl. When the carbonate had all dissolved, the solution was boiled vigorously to expel dissolved CO_2. It was then cooled and diluted. Before being made up to volume the pH was adjusted to 7.3 by means of 0.1 N

[2] It may, however, be mentioned that the direct precipitation of a substance with the formula $Ca_3(C_6H_5O_7)_2$ would require a fifth order reaction. Further investigation may reveal interesting similarities between tricalcium citrate and tricalcium phosphate. (*Cf.* Paper III in this series.)

NaOH with phenol red as indicator. The sodium chloride when dissolved in water gave an acid reaction;[3] before being made up to volume the pH was adjusted to 7.3 as in the case of the $CaCl_2$. The sodium citrate was found to require very little addition of 0.1 N HCl for attainment of pH 7.3. In making up the solution the formula $Na_3C_6H_5O_7 \cdot 5\frac{1}{2}H_2O$ or 2 $Na_3C_6H_5O_7 \cdot 11\ H_2O$ was employed.

The experimental data are given in terms of observed resistances. The same cell was used throughout this investigation; the specific resistance in each case may therefore be calculated from the observed resistance and the cell constant, which had a value of 0.519.

TABLE I.

Conductivity Titration of 5.0 Cc. of 0.00125 N NaCl with 0.005 N Sodium Citrate.

Na citrate added. (1)	Total volume. (2)	$R_{obs.}$ (3)	$R_{calc.}$* (4)
cc.	*cc.*	*ohms*	*ohms*
0.0	5.0	848	
0.5	5.5	848	853
1.0	6.0	851	857
1.5	6.5	853	860
2.0	7.0	854	863
3.0	8.0	857	867
4.0	9.0	860	871
5.0	10.0	861	874

Observed resistance of the sodium citrate solution = 899 ohms.

*$R_{calc.} = \dfrac{v_1R_1 + v_2R_2}{v_1 + v_2}$.

Normal Conductivity Titration.—The measured resistance of the NaCl solution was 848 ohms; that of the sodium citrate solution was 899 ohms. 5 cc. of the NaCl solution were placed in the cell and then 0.5 cc. of the sodium citrate solution was added. The mixture was stirred and the resistance of the mixture determined. Another 0.5 cc. of the sodium citrate solution was then added, and the resistance again measured after the mixture was stirred. The addition of aliquots of the sodium citrate solution and the measurement of the resistances of the resulting mixtures were

[3] In subsequent experiments which we have performed at 38° we have used fused NaCl. The fused salt gives a neutral solution in water.

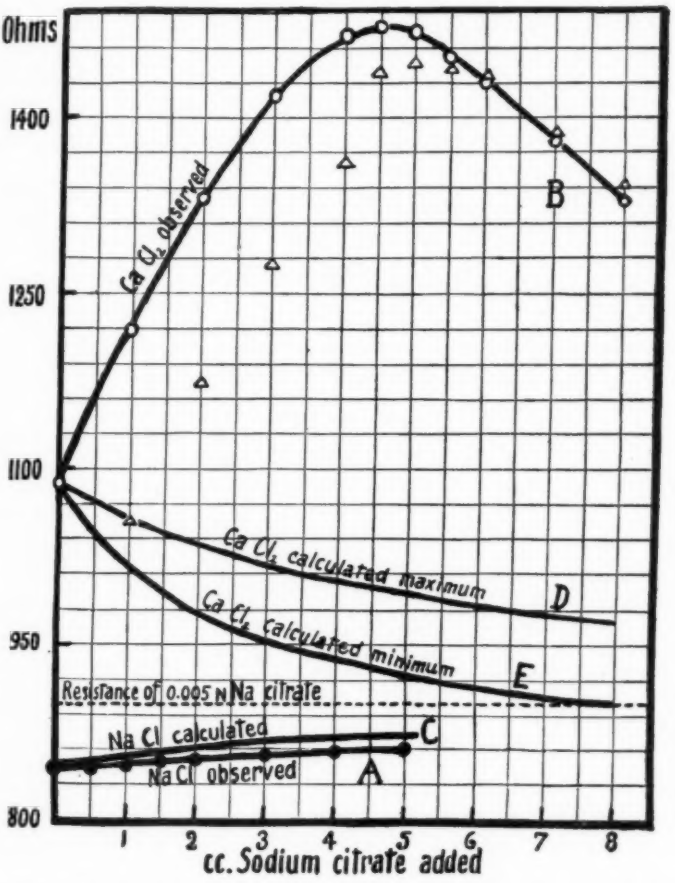

Fig. 1. Conductivity titrations of 0.00125 N NaCl (●) with 0.005 N Na citrate and of 0.005 N $CaCl_2$ (○) with 0.005 N Na citrate. △ signifies point to point calculation.

continued until the total volume was 10.0 cc.; *i.e.*, until 5.0 cc. of sodium citrate had been added to the original 5.0 cc. of NaCl. The data are given in Table I and are shown graphically by Curve A in Fig. 1. These values are normal values; *i.e.*, the resistances of the mixtures are intermediate between those of the two original solutions, the resistance of each mixture depending upon the relative quantities of the original solutions in that mixture.

Abnormal Curve.—This same procedure was repeated with 0.005 N $CaCl_2$ instead of NaCl. The resistance of the $CaCl_2$ solution

TABLE II.

Conductivity Titration of 5.0 Cc. of 0.005 N $CaCl_2$ with 0.005 N Sodium Citrate.

Na citrate added.	Total volume.	$R_{obs.}$	Calculated resistance.			
			Maximum.	Minimum.	Point to point.	
(1)	(2)	(3)	(4)	(5)	(6)	(3) − (6)
cc.	*cc.*	*ohms*	*ohms*	*ohms*	*ohms*	*ohms*
0.0	5.0	1089	1089	1089	1089	
1.0	6.0	1220	1057	1014	1057	+163
2.0	7.0	1332	1035	979	1174	+158
3.0	8.0	1418	1018	955	1278	+140
4.0	9.0	1468	1005	937	1360	+108
4.5	9.5	1474	999		1438	+ 36
5.0	10.0	1470	994	925	1445	+ 25
5.5	10.5	1454	989		1443	+ 11
6.0	11.0	1428	985	914	1429	− 1
7.0	12.0	1380	978	905	1384	− 4
8.0	13.0	1330	972	899	1343	− 13

$R_{obs.}$ of 0.005 N Na citrate = 899 ohms.

was 1089 ohms, and that of the 0.005 N sodium citrate solution was 899 ohms. 5 cc. of the $CaCl_2$ solution were placed in the cell and the resistance measured; it was 1089 ohms. Then 1 cc. of the sodium citrate solution was added; the resistance after this addition was 1220 ohms. Addition of another cc. of sodium citrate caused the resistance to change to 1332 ohms, *etc.* The data are given in Table II and are shown graphically by Curve B in Fig. 1.

The resistance of the citrate solution is less than that of the $CaCl_2$ solution. On addition of sodium citrate to the latter the resistance of the resulting mixture should be *lower* than that of the $CaCl_2$; it should be intermediate between those of the two

solutions mixed. It is seen, however, that the resistance of the mixture is *higher* than that of the $CaCl_2$, and it continues to increase until approximately an equal volume of the citrate solution has been added. After about an equal volume of sodium citrate has been added, then and then only, does the resistance decrease as expected.

The concentration of 0.005 N Ca^{++} was selected because this is the concentration of calcium in normal serum, *i.e.* 10 mg. per 100 cc. This behavior with citrate is, however, not confined to this concentration of sodium citrate. A similar conductivity titration was performed with $\frac{N}{167}\left(\frac{M}{500}\right)$ sodium citrate; a similar curve was obtained showing a marked increase in resistance to a maximum at about 4 cc. of added sodium citrate, followed by a decrease in resistance on further addition of citrate solution.

The same type of curve was also obtained when 0.02 N $CaCl_2$ was titrated with $\frac{N}{33}\left(\frac{M}{100}\right)$ sodium citrate. The experiments with these various concentrations were performed repeatedly; similar results were obtained in all cases.

Calculated Titration Curves.—The resistance of a mixture of two solutions with resistances R_1 and R_2 respectively, was calculated from the expression

$$R_{\text{mixt.}} = \frac{v_1R_1 + v_2R_2}{v_1 + v_2} \qquad (1)$$

where v_1 and v_2 are the volumes of the two solutions taken for a particular mixture. The resistances so calculated for mixtures of NaCl and sodium citrate are given in Column 4 of Table I. They are also shown graphically by Curve C in Fig. 1. It is seen that the theoretical resistances calculated in this way do not differ by more than 1 or 2 per cent from the observed values. The calculated values are all high, however, and the divergence from the observed values increases gradually as the total volume increases.

The theoretical titration curve for $CaCl_2$ and sodium citrate was also calculated by means of equation (1). The values of the resistances calculated in this way are given in Table II, Column 4; they are also given graphically by Curve D in Fig. 1.

These calculations do not take into consideration the change

produced in the activity coefficient of each salt by the increase in volume. When a given salt solution is mixed with an equal volume of water, the resistance of the dilute solution is not half of the resistance of the original solution but is less than half. This is due to an increase in the activity coefficient or, according to the classical theory, to an increase in the "degree of ionization."

Ruby and Kawai (9) employed several methods of calculating the conductances of mixtures of two solutions from the conduct-

TABLE III.

Conductivity Titration of 5.0 Cc. of 0.005 N $CaCl_2$ with Water.

Water added. (1)	Total volume. (2)	$R_{obs.}$ (3)	$R_{calc.}$* (4)	Resistance of 0.005 N $CaCl_2$ corrected for dilution effect.† (5)
cc.	*cc.*	*ohms*	*ohms*	*ohms*
0.0	5.0	1089	1089	1089
1.0	6.0	1282	1307	1068
2.0	7.0	1472	1525	1051
3.0	8.0	1656	1742	1035
4.0	9.0	1838	1980	1021
5.0	10.0	2020	2178	1010
6.0	11.0	2197	2396	999
7.0	12.0	2368	2614	987
8.0	13.0	2543	2831	978

* Calculated, neglecting increased degree of ionization, by means of the equation

$$R_{calc.} = \frac{v_1 + v_2}{v_1} \times 1089$$

where v_1 = volume of salt solution and v_2 = volume of added water.

† Corrected resistance $= \frac{R_{obs.}}{R_{calc.}} \times 1089.$

ances of the original solutions. For pairs of uni-univalent electrolytes they obtained good agreement between observed results and the calculated values. Renholm (10) applied a modification of the expression of Debye and Hückel (11) to the calculation of conductances of mixtures of KCl and $LaCl_3$. He obtained almost exact agreement between the values calculated by the modified Debye-Hückel equation and those calculated by the simple rule of mixtures. Our calculated resistances have been obtained according to the simple rule of mixtures.

Since it is uncertain to what extent the theory of complete dissociation of strong electrolytes applies to citrates, we determined the approximate effect of dilution on the change in the activity coefficient of each salt by means of conductivity titrations, performed as follows:

The same volume of 0.005 N $CaCl_2$ was taken as in the titration with sodium citrate, *i.e.* 5.0 cc. Then water was added, 1.0 cc. at

TABLE IV.

Conductivity Titration of 5.0 Cc. of Water with 0.005 N Sodium Citrate.

Na citrate added. (1)	Total volume. (2)	$R_{obs.}$ (3)	$R_{calc.}$* (4)	Resistance of 0.005 N Na citrate corrected for dilution effect.† (5)
cc.	*cc.*	*ohms*	*ohms*	*ohms*
0.0	5.0			
1.0	6.0	4450	5340	742
2.0	7.0	2796	3115	799
3.0	8.0	2188	2373	821
4.0	9.0	1874	2003	833
5.0	10.0	1680	1780	840
6.0	11.0	1547	1632	844
7.0	12.0	1452	1586	847
8.0	13.0	1382	1446	850

Resistance of 0.005 N Na citrate = 899.

* Calculated, neglecting increased degree of ionization, by means of the equation

$$R_{calc.} = \frac{v_1 + v_2}{v_1} \times 899$$

where v_1 = volume of salt solution and v_2 = 5.0 cc. of water.

† Corrected resistance = $\frac{R_{obs.}}{R_{calc.}} \times 899$.

a time, instead of sodium citrate solution. The solution was stirred and the resistance was measured in the usual way after each addition of water. The results are given in Table III.

To determine the effect of dilution on the resistance of the sodium citrate solution, 5.0 cc. of water were titrated with sodium citrate. Instead of $CaCl_2$ being used, 5.0 cc. of water were taken initially. To this was added 0.005 N sodium citrate, 1.0 cc. at a time. The resistance was determined after the addition of each aliquot. The results are given in Table IV.

Column 3 in Table III gives the observed resistances and Column 4 gives the resistances calculated without consideration of the increase in activity coefficient with increasing dilution. It is seen that the observed resistances are lower than those calculated in this way and that the differences are greater the more dilute the solution. The calculations in Column 4 are made on the simple assumption that the resistance of a mixture is directly proportional to the total volume and inversely proportional to the volume of added salt solution; *i.e.*, if a given solution has a given resistance, then doubling the volume, by the addition of water, will result in doubling the resistance. The expression used is

$$R_{\text{calc.}} = \frac{v_1 + v_2}{v_1} \times (\text{resistance of the undiluted salt solution}) \quad (2)$$

where v_1 is the volume of salt solution and v_2 is the volume of added water in a given mixture.

Column 5, Table III, gives the resistance of the $CaCl_2$ solution corrected for the effect of dilution on the activity coefficient. For example, a solution of $CaCl_2$ with a measured resistance of 1089 ohms, when diluted with an equal volume of water would be expected to have twice the resistance, or 2178 ohms, if the "degree of ionization" did not increase with dilution. When 5 cc. of this $CaCl_2$ solution are diluted with 5 cc. of water, the measured resistance is 2020 ohms instead of 2178 ohms; *i.e.*, the resistance is less than that calculated by means of equation (2). In order that the resistance calculated by equation (2) shall be equal to that experimentally observed, the resistance of the 0.005 N $CaCl_2$ solution is corrected by a different factor for each dilution. This is shown in the following equation

$$\text{Corrected resistance} = \frac{R_{\text{obs.}}}{R_{\text{calc.}}} \times 1089^* \quad (3)$$

* The corrected resistance may be obtained without first obtaining $R_{\text{calc.}}$. The expression used is

$$\text{corrected resistance} = \frac{v_1}{v_1 + v_2} \times R_{\text{obs.}}$$

For equation (3), $R_{\text{obs.}}$ is taken from Column 3, Table III, and $R_{\text{calc.}}$ is taken from Column 4. The corrected resistance so obtained is given in Column 5.

An analogous procedure was employed with the data for sodium citrate; the values are given in Table IV.

Theoretical Curves for the Conductivity Titration.—The purpose of all these dilution experiments and calculations was to determine the position and shape the theoretical titration curve would have if no chemical reaction occurred when sodium citrate is added to calcium chloride. In Column 4, Table II, are given the theoretical resistances calculated from equation (1) with the value of 1089 ohms for the $CaCl_2$ and 899 ohms for the sodium citrate. These theoretical titration values are shown by Curve D in Fig. 1. Inasmuch as the effect of dilution on the increase in the activity coefficient of each salt was neglected, this curve gives not the exact expected resistances, but the probable maximum resistances of mixtures of these two solutions.

Another set of theoretical titration values was calculated also by means of equation (1), but with the values given in Column 5 of Tables III and IV; *i.e.*, with the resistances corrected for dilution. This second set of theoretical titration values is given in Column 5, Table II, and is given graphically by Curve E in Fig. 1. The presence of sodium citrate diminishes somewhat the activity coefficient of the calcium chloride, and the presence of calcium chloride diminishes somewhat the activity coefficient of the sodium citrate; these effects have not been allowed for. Curve E, therefore, does not give the exact position of the curve, but rather the probable minimum position of the curve for mixtures of these two solutions. If no chemical reaction occurred when sodium citrate is added to calcium chloride, the resistances of mixtures of the two solutions would probably lie somewhere between Curves D and E. *The experimental Curve B varies markedly in position from even the highest expected curve; moreover it possesses a maximum, while the theoretical curves have no point of inflection.*

Point to Point Resistances.—It is seen that not only are the experimental resistances greater than the highest values calculated, but that there is also a maximum in the experimental curve. The experimental curve was investigated by means of point to point calculations.

In these point to point calculations, equation (1) was employed, but the values R_1, v_1, and v_2 have different significance. The resistance of a given mixture is calculated from the resistance and

volume of the preceding mixture. The calculated resistance of the mixture of 5.0 cc. of $CaCl_2$ and 1.0 cc. of sodium citrate is the same as is given in Column 4 of Table II; *i.e.*, 1057 ohms. For the next point, with a total volume of 7.0 cc., R_1 is taken as the *measured* resistance of the preceding mixture of 6.0 cc. total volume, while v_1 is 6.0 cc. In all of this series of calculations $R_2 = 899$ ohms and $v_2 = 1$ cc. For this second mixture therefore, the expression

$$R_{calc.} = \frac{R_1 v_1 + R_2 v_2}{v_1 + v_2}$$

becomes

$$R_{calc.} = \frac{(6 \times 1220) + (899 \times 1)}{6 + 1} = 1174 \text{ ohms}$$

For the third mixture (total volume = 8 cc.), R_2 is taken as the measured resistance of the second mixture. This gives

$$R_{calc.} = \frac{(7 \times 1332) + (899 \times 1)}{7 + 1} = 1278 \text{ ohms}$$

The resistances calculated in this way are given in Column 6 of Table II.

The differences between the point to point calculated resistances and the measured resistances are given in Column 7 of Table II. These differences are large for the first additions of sodium citrate to calcium chloride. As more sodium citrate is added, the difference between what is expected and what is observed diminishes; when 5.5 cc. have been added, the difference is less than 1 per cent, *and the observed and calculated resistances agree from this point on to within 1 per cent.*

DISCUSSION.

We are interested here in the changes in resistance which occur when these solutions are mixed rather than in the actual resistances themselves. The resistances given in Tables I and II for the three original solutions are not those of pure solutions but of solutions containing some NaCl in addition, since the original solutions were all adjusted to the same pH with either NaOH or

HCl. The position of the titration curves with respect to the Y axis varies with the amount of additional NaCl; the shapes of the curves, however, are not affected, or are only slightly affected, by the presence of NaCl.

In the control titration of NaCl with sodium citrate, the observed results agree well with the theoretical. The resistance of the citrate solution is greater than that of the NaCl solution. Mixtures have resistances as expected; *i.e.*, intermediate between those of the two original solutions. Moreover, the resistance of any given mixture agrees within about 1 per cent with that calculated from equation (1). The greater the volume of added sodium citrate, the greater is the calculated resistance, and also that of the observed resistance—there is no maximum or minimum in either the theoretical curve, Curve C, or the experimental curve, Curve A.

For mixtures of calcium chloride and sodium citrate, a similar theoretical curve, Curve D, was obtained. In this theoretical curve, the resistances of mixtures decrease progressively with increasing proportions of sodium citrate solution. The experimental curve, Curve B, however, is strikingly different from the theoretical—addition of sodium citrate causes the resistance to increase instead of decrease. The resistance continues to increase to a maximum; only after about an equal volume of the citrate solution has been added, does the resistance decrease as expected. For a mixture containing 5.0 cc. of each solution, the calculated resistance from Curve D is 994 ohms while the observed resistance is 1470 ohms; *i.e.*, the observed resistance is about 50 per cent higher than the theoretical. If we employ the other theoretical curve, Curve E, the difference is still more marked and is in the neighborhood of 60 per cent.

The solutions were adjusted to the same pH before the titration was begun; at the end of the titration, the pH of the mixture was still 7.4. The peculiar observed increase in resistance to a maximum cannot, therefore, be attributed to withdrawal of hydrogen and hydroxyl ions from the solution because of the reaction

$$H^+ + OH^- \rightarrow H_2O$$

There seems to be but one possible cause of this observed increase in resistance; namely, a decrease in the number of ions. Mixing

sodium ions and citrate ions with calcium ions and chloride ions, all at the same pH, apparently is followed by a decrease in the total number of ions. This ionic reaction cannot be due to the effect of the sodium citrate on the chloride since normal results are obtained when the sodium citrate solution is added to one of sodium chloride. The reaction must therefore involve the calcium ions.

Calcium ions appear therefore to be removed from solution by addition of sodium citrate. The nature of the calcium citrate complex which is so formed cannot be determined without further study. It is possible that calcium ion unites with citrate ion to form a neutral compound or a complex negatively charged ion, or a complex positively charged ion. Apparently all of the calcium can be bound in this way as evidenced by the point to point calculated resistances, given in Table II. The observed resistances for the first additions of sodium citrate are much higher than the resistances calculated in this way; as more citrate is added, the discrepancies diminish. After an equal volume of citrate has been added, the observed and calculated point to point resistances agree to within 1 per cent. (See also Fig. 1.)

This means that when sodium citrate is first added to a solution of calcium ions, an ionic reaction occurs which results in a decrease in the number of ions. With further addition of sodium citrate, fewer and fewer ions are removed from solution. When the reaction is complete, further addition of sodium citrate does not cause any further diminution in the number of ions; the resistance from this point on therefore decreases in agreement with the calculated values.

SUMMARY.

1. Conductivity titration of sodium chloride with sodium citrate at 25° gives results which are normal, and which are in good agreement with calculated values.

2. Similar conductivity titrations of calcium chloride with sodium citrate give abnormal results. The observed resistance, instead of decreasing, increases to a maximum; after the maximum is passed it decreases in agreement with theory.

3. These results are direct evidence for the binding of calcium ions by sodium citrate in some kind of soluble complex.

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THE ELECTRICAL TRANSFERENCE OF CALCIUM IN BLOOD SERUM PROTEIN SOLUTIONS.

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INTRODUCTION.

In a paper by Updegraff, Greenberg, and Clark (1) there was stated "Rona and Takahashi [(2)], in 1911 and 1913, suggested that the non-diffusible calcium was bound to the proteins of the serum and this was tacitly accepted by all later investigators until the publication by Cameron and Moorhouse [(3)], in 1925, of their paper 'Tetany of Parathyroid Deficiency.' In this paper Cameron and Moorhouse rejected the above hypothesis and proposed instead that the non-diffusible calcium is bound to some organic substance which may be the parathyroid hormone."

Since then experimental work to demonstrate that the non-diffusible calcium is chiefly held in the form of a Ca-protein complex ion has been published by Marrack and Thacker (4), Loeb (5), and Loeb and Nichols (6). Of these studies the most complete is that of Loeb and Nichols. By membrane distribution experiments, these authors have shown that the amount of calcium inside of sacs containing serum or serum proteins was from 30 to 50 per cent higher than would be expected from the distribution ratios of chloride ion in dialysate and serum.

In the present work the attempt is made to demonstrate the existence of complex Ca-protein ions by the electrical transference method.

EXPERIMENTAL.

The experimental work was carried out on electrodialyzed beef serum. To decrease the time of centrifuging and obtain

* Aided by a grant from the Research Board of the University.

XUM

a complete separation of the serum, the blood was made somewhat hypertonic by the addition of a 0.1 volume of 1.4 per cent sodium chloride solution. About 1 liter of such a serum was placed in the middle compartment of a dialyzer and dialyzed electrolyte-free, until the specific conductivity fell to from 1 to 2 $\times$ 10^{-5} mhos. The dialyzer was made by sawing a 6 volt, rubber, automobile battery case into three sections. The membranes were a parchment cathode and chrome gelatin anode as described by Freundlich and Farmer-Loeb (7). The pH of the electrodialyzed serum was found to be between 5.6 and 5.7. The dialyzed serum was kept in a refrigerator under toluene and samples were withdrawn for use as needed.

The analytical methods employed were the following.

The protein was determined by drying 10 gm. portions of solution in porcelain crucibles at 105° and weighing. From this weight was subtracted the weight of the cation in the solution. The titratable base in the solutions, which in these experiments represents the sum of the sodium and calcium present, was determined by electrometric titration to pH 5.6 with the hydrogen 0.1 N calomel electrode, 0.025 N HCl being used. Schmidt and Hoagland's tables (8) were used to obtain the pH values from the E. M. F. readings. The calcium itself was determined by the Van Slyke and Sendroy (9) gasometric method.[1] The amount of sodium present was obtained by difference.

The transference experiments were carried out in duplicate, at 25°, in the same kind of cells and with the same technique as were previously used in casein transport number studies (10). In these solutions, unlike that of casein, there was no adhering deposit of protein to the platinum anode. This made it necessary to calculate the transport numbers of the proteins of the solutions in a different manner than heretofore. The method employed was to assume the equivalent weight of the protein to be the reciprocal of the equivalents of base added per gm. of protein. The amount of protein gained by the anode, calculated in equivalents, divided by the equivalents of current passed through the solution gives the transference number. Since there are a number of proteins in the solution, no significance can be placed on the

[1] I am indebted to Mr. H. E. Ballard for carrying out the calcium analyses.

absolute value of the transport numbers calculated in this way. However, it seems that the calculated transport numbers should bear some constant relationship to the true transport numbers of the protein.

DISCUSSION.

In an attempt to get a picture of the carrying of the current in its simplest possible terms, the first measurements were made on

TABLE I.

Transport of Electrodialyzed Serum in a Single Alkali Solution.

Serum sample.	Serum proteins.	Alkali per gm. of protein.	pH	T_{serum^-}.	T_{cation^+}.
		Alkali, NaOH.			
	per cent	*m.-eq.*			
S_2	2.0	0.28			0.60
	2.0	0.28			0.57
	1.2	0.24	7.9	0.25	
	1.2	0.24	7.9	0.25	
S_3	3.5	0.23	7.9	0.18	0.70
	3.5	0.23	7.9	0.19	0.61
S_4	3.3	0.25	8.2	0.23	
	3.3	0.25	8.2	0.21	
Average				0.22	0.62
		Alkali, $Ca(OH)_2$.			
S_2	3.2	0.26	8.2	0.32	
	3.2	0.26	8.2	0.32	
	3.2	0.27	8.8	0.27	0.48
	3.2	0.27	8.8	0.27	0.55
S_3	4.4	0.22	8.0	0.21	0.75
	4.4	0.22	8.0	0.20	(0.95)?
Average				0.27	0.60

solutions to which either NaOH or $Ca(OH)_2$ only had been added. The results of these measurements are given in Table I. From Table I it is to be seen that sodium and calcium ions carry about the same fraction of the current. From the mobilities of the sodium and calcium ions, which are 50 and 60 mhos respectively at 25°, it would be expected that the transference numbers of

these ions should be in that ratio, providing there are no complicating factors. In this instance, due to the formation of a complex ion of protein and calcium, some calcium will be carried in the opposite direction to its normal movement. As a result the

TABLE II.

Electrical Transference of Electrodialyzed Serum in Mixed Solutions of Sodium and Calcium Hydroxide.

Serum sample.	Serum proteins.	Base added.		pH	Ratio of $Ca(OH)_2$: Titratable base*	T serum proteins.	T titratable base.*	T ½ Ca^{++}.		Loss of Ca from anode.	
		NaOH per gm. protein.	$Ca(OH)_2$ per gm. protein.					Obtained.	Calculated.	Found.	Calculated.
	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)
	per cent	*m.-eq.*	*m.-eq.*		*per cent*					*mg.*	*mg.*
S_3	4.00	0.195	0.055	8.0	22	0.23		(−0.08)	0.15	(−1.15)	2.2
	4.00	0.195	0.055	8.0	22	0.24		(−0.08)	0.15	(−0.65)	1.3
S_4	3.45	0.226	0.07	9.3	24	0.31	0.52	(−0.09)	0.16	(−0.65)	1.2
	3.45	0.226	0.07	9.3	24	0.32	0.51	(−0.11)	0.16	(−0.50)	0.8
	3.49	0.135	0.128	8.8	49	0.29	0.49	(−0.02)	0.33	(−0.05)	2.0
	3.49	0.135	0.128	8.8	49	0.30	0.55	0.0	0.33	0.0	1.3
	3.43	0.204	0.048	8.7	19	0.28		0.0	0.13	0.0	1.1
	3.43	0.204	0.048	8.7	19	0.27		0.01	0.13	(−0.1)	0.7
	3.67	0.174	0.09	8.6	34	0.31	0.62	0.0	0.23	0.0	1.6
	3.67	0.174	0.09	8.6	34	0.31	0.49	0.03	0.23	0.3	1.0
S_5	4.22	0.168	0.079	8.3	32	0.27		0.0	0.21	0.0	1.5
	4.22	0.168	0.079	8.3	32	0.25		(−0.02)	0.21	(−0.1)	1.0
	3.70	0.199	0.088	8.4	30	0.29		0.03	0.20	0.15	1.9
	3.70	0.199	0.088	8.4	30	0.30		0.05	0.20	0.15	1.2
	4.26	0.184	0.048		21	0.23	0.63	0.04	0.14	0.3	1.1
	4.26	0.184	0.048		21	0.21	0.66	0.04	0.14	0.2	0.8
Average.....						0.28	0.56				

* Titratable base represents the sum of sodium and calcium.

calculated transport numbers will be lower depending upon the amount of the complex ion, and may even become negative in sign. The amount of complex formation that might be expected, with the non-diffusible calcium (1) as a measure, is not over 0.04 milli-equivalents of calcium per gm. of protein. Since this is

less than 20 per cent of the total calcium in the above solutions, it is not surprising that it does not show to any great degree in the transference results of the solutions containing only calcium hydroxide.

To attempt to reduce the concentration of calcium so that if complex ions were present they would have a significant effect on the calcium transference, experiments were next carried out with solutions of electrodialyzed serum containing mixtures of sodium and calcium hydroxide. The results of these experiments are given in Table II. The column headings are self-explanatory. In Columns 8 and 9 are given the transport numbers of calcium as actually determined by analysis, and as calculated from the concentration of calcium in the solution and its mobility, no complex ion formation being assumed. For the calculation of the expected transport number of the calcium, the average figure of the titratable base transport, 0.56, was taken as a basis. This figure multiplied by the ratio of calcium to titratable base in the solution (Table II, Column 5) and corrected for the difference in mobility by multiplying by $\frac{60}{50}$ gives the expected calcium transport number, with the assumption of no complex ion formation. It is to be seen that the transport numbers found in all cases diverge widely from the calculated numbers and in some cases are even opposite in sign. To show that errors in analysis cannot account for the low transference numbers obtained, there are given in the last two columns the found and calculated loss of calcium from the anode compartment for the experiments, in mg. of calcium. The maximum possible error of analysis would be about 0.2 to 0.3 mg. The difference between the found and calculated loss is usually many times this amount.

The results, then, indicate that there are complex ions of calcium and the serum proteins in their respective solutions. This is in harmony with the findings of Greenberg and Schmidt (11) and of Kirk and Schmidt (12) on solutions of casein in the alkali earth hydroxides. The amount of complex ion formation with the serum proteins is much less, however, than with casein.

SUMMARY.

By the electrical transference method, the presence of complex Ca-protein ions was demonstrated in solutions of electrodialyzed

blood serum to which mixtures of sodium and calcium hydroxide were added.

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STUDIES OF GAS AND ELECTROLYTE EQUILIBRIA IN BLOOD.

XII. THE VALUE OF pK′ IN THE HENDERSON-HASSELBALCH EQUATION FOR BLOOD SERUM.

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Hasselbalch in 1917 proposed the now widely applied calculation of the blood pH from the CO_2 content and tension by means of Henderson's (1909) equation in the logarithmic form

$$(1) \qquad pH = pK' + \log \frac{[BHCO_3]}{[H_2CO_3]}$$

In this equation pK′ is the negative logarithm of the apparent dissociation constant, K', of the more acid hydrogen atom of H_2CO_3. K' is a collective constant. It includes not only the first acid dissociation constant of the acid, H_2CO_3, but also the equilibrium constant of the reaction, $CO_2 + H_2O = H_2CO_3$, the activity coefficient of the HCO_3 anion, and the dissociation coefficient of $BHCO_3$, in case its dissociation is not complete.

The value of pK′ was determined by Hasselbalch (1917) for solutions containing $NaHCO_3$ and H_2CO_3 in the range of concentrations found in the blood, and was found to be about 6.1 at 38°, when both H_2CO_3 and $BHCO_3$ were expressed as molal concentrations. Warburg (1922) studied the value of pK′ further in salt solutions; and Hastings and Sendroy (1925) showed that pK′ increased in a linear manner with the square root of the ionic strength of the salt solutions studied, in accordance with the theory of Debye and Hückel. Van Slyke, Hastings, Murray, and Sendroy (1925) also showed that the pK′ value for serum

approximates that of a salt-bicarbonate solution of similar ionic strength.

In whole blood Hasselbalch (1917) found that pH values calculated with the same pK′ constant that he had determined in bicarbonate solutions approximated the pH values found with the hydrogen electrode. Parsons (1917) demonstrated that the pH determined electrometrically in whole blood is really that of the serum, the suspended corpuscles having no direct influence on the results. Since the $BHCO_3$ content per unit volume of the corpuscles is much less than that of the serum, and to a degree variable with the pH, any pK′ value for whole blood must contain an added, variable factor, to correct for the unequal $BHCO_3$ distribution. The effect of hemoglobin content, degree of oxygenation, and serum pH on this factor has been studied theoretically and experimentally by Warburg (1922), by Van Slyke, Wu, and McLean (1923), and by Peters, Bulger, and Eisenman (1923). The number of factors affecting the value of of pK′ in whole blood indicates the desirability, in estimating serum pH by Hasselbalch's method, of making the CO_2 determinations when possible on serum rather than whole blood.

The data in the literature from which the pK′ for blood serum can be calculated have been reviewed by Warburg (1922), and more recently by Cullen, Keeler, and Robinson (1925). There still exists a considerable divergence in results concerning the figure in the second place decimal of the pK′ for serum, which we shall designate as pK_s'. The purpose of the present paper is to add to the available data a number of determinations on human sera, on which not many values have been reported, and to summarize for comparison the results obtained by other investigators with the more modern methods.

The value of pK′ is calculated from experimentally determined values of pH and the molal concentrations, $[BHCO_3]$, $[H_2CO_3]$, according to the rearranged Hasselbalch equation,

$$(2) \qquad pK' = pH + \log [H_2CO_3] - \log [BHCO_3]$$

In calculating the value of pK′ the solubility coefficient of CO_2 plays an important part not indicated by the above equation. The value of $[H_2CO_3]$ has in all published determinations been fixed by saturating the solution with CO_2 under a known tension,

and estimating the H_2CO_3 as the physically dissolved CO_2, according to Henry's law, expressed in the equation

$$(3) \qquad [H_2CO_3] \text{ in cc. } CO_2 \text{ per 100 cc. solution} = \alpha \times \frac{p}{760}$$

or

$$(4) \qquad [H_2CO_3] \text{ in mM per liter solution} = \frac{\alpha\, p}{22.26 \times 0.76} = 0.0591\ \alpha\, p$$

where p is the CO_2 pressure in mm., and α is Bunsen's solubility coefficient, representing the cc. of CO_2, measured at 0°, 760 mm., that are dissolved by 1 cc. of solution when under 760 mm. pressure of the gas. As a rule, the value of $[BHCO_3]$ has been determined by finding the total CO_2 content of the solution, and subtracting therefrom the H_2CO_3. The equation expressing the calculation of pK′ from the directly determined values therefore is:

$$(5) \qquad pK' = pH + \log 0.0591 + \log \alpha + \log p - \log ([CO_2] - 0.0591\, \alpha\, p)$$

where $[CO_2]$ represents total CO_2 content of the solution in millimols per liter.

It is evident from the third term of the right hand member that the value of pK′ will vary in direct proportion to the logarithm of the value used for the solubility coefficient, α. The latter also influences the last term, but with the relative proportions of total and dissolved CO_2 found in serum the effect of α on pK′ through its influence on the last term is not significant.

In a recent paper from this laboratory (Van Slyke, Sendroy, Hastings, and Neill (1928)), the value of α for serum has been directly determined for the first time, and found to be 0.510. It is used for the calculation of pK_s' from the data given below, and for recalculation of similar data of other authors in the literature.

Methods.

Samples of human serum were equilibrated with mixtures of air and CO_2 in double chambered tonometers according to the "First Saturation Method" of Austin *et al.* (1922).

The total CO_2 content of the saturated serum was determined in the manometric apparatus of Van Slyke and Neill (1924) the

TABLE I.

Determinations of pK_s' Value of Human Sera.

Subject and serum No.	H_2O content.	Total CO_2 content.		CO_2 tension.	H_2CO_3 content.*		$BHCO_3$ content.		*E* corrected to 760 mm. H_2 pressure.	E_0	pH	pK_s'	
												From data in mM per liter.	From data in mM per kg. H_2O.
	gm. per cc.	*mM per l.*	*mM per kg. H_2O*	mm.	*mM per l.*	*mM per kg. H_2O*	*mM per l.*	*mM per kg. H_2O*	*millivolts*	*millivolts*			
Normal. H–1	0.923	27.73	30.04	34.8	1.048	1.138	26.68	28.90	698.4	235.1	7.510	6.105	6.105
H–2	0.923	30.52	33.07	58.9	1.773	1.927	28.75	31.14	686.0	235.2	7.308	6.098	6.100
Normal. O–1	0.926	23.92	25.83	36.1	1.087	1.181	22.83	24.65	694.1	235.1	7.441	6.119	6.122
O–2	0.926	26.41	28.52	60.4	1.818	1.976	24.59	26.54	680.9	235.2	7.225	6.094	6.097
Normal. W–1	0.930	28.94	31.12	36.2	1.090	1.184	27.85	29.94	698.1	235.2	7.503	6.096	6.100
W–2	0.930	31.38	33.74	61.4	1.848	2.008	29.53	31.73	686.3	235.2	7.313	6.110	6.114
Rheumatic. M–1	0.927	27.35	29.50	36.1	1.087	1.181	26.26	28.32	696.9	235.1	7.486	6.103	6.106
M–2	0.927	29.69	32.03	61.1	1.840	1.998	27.85	30.03	684.5	235.1	7.285	6.105	6.108
Rheumatic. H–1	0.918	29.64	32.29	36.4	1.096	1.190	28.54	31.10	698.7	235.1	7.515	6.097	6.098
H–2	0.918	32.09	34.96	61.2	1.843	2.001	30.25	32.96	686.5	235.1	7.318	6.103	6.102
Nephritic. G–1	0.927	31.08	33.53	36.3	1.093	1.187	29.99	32.34	701.1	235.2	7.553	6.115	6.118
G–2	0.927	33.88	36.55	59.7	1.800	1.952	32.08	34.60	689.9	235.2	7.371	6.120	6.122

	G–2.....	0.927	33.88	36.55	59.7	1.800	1.952	32.08	34.60	689.9	235.2	7.371	6.120	6.122
Nephritic.	V–1.....	0.943	28.12	29.82	34.9	1.051	1.142	27.07	28.68	698.8	235.3	7.514	6.103	6.114
	V–2.....	0.943	29.85	31.65	58.4	1.759	1.910	28.09	29.74	685.4	235.0	7.301	6.098	6.108
Nephritic.	C–1.....	0.939	28.75	30.62	33.7	1.014	1.102	27.74	29.52	701.1	235.3	7.551	6.114	6.123
	C–2.....	0.939	30.65	32.64	57.2	1.722	1.871	28.93	30.77	688.4	235.0	7.350	6.124	6.134
Average of all sixteen sera.....													6.105	6.111
" " ten non-nephritic sera.....													6.103	6.105

* H_2CO_3 in *mM per liter* is calculated as $[H_2CO_3] = \frac{\alpha\, p}{22.26 \times 0.76} = 0.0301p$, where p represents CO_2 tension, and the value taken for the solubility coefficient, α, is 0.510, found for normal serum by Van Slyke, Sendroy, Hastings, and Neill (1928).

In *mM per kg.* H_2O, $[H_2CO_3] = \frac{\alpha^0\, p}{22.26 \times 0.76} = 0.0327p$, where α^0 representing the cc. of CO_2 dissolved *per gm.* H_2O in serum under 1 atmosphere of CO_2 pressure, has the value 0.553, found for normal serum by the same authors.

results being calculated by the revised factors of Van Slyke and Sendroy (1927).

The CO_2 content of the gas phase in the tonometer at the end of equilibration was determined by Y. Henderson's modification (1918) of the Haldane apparatus for air analysis.

The water contents of the sera were determined by drying 2 cc. samples to constant weight at 110°.

The pH was determined at 38° with the hydrogen electrode. The rocking electrode chamber devised by Clark (1915) was employed, with the modification introduced by Cullen (1922) to permit the insertion of a thermometer. The system used and the standardization with 0.1 N HCl have been described on pages 708 and 709 of Paper VIII of this series (Van Slyke, Hastings, Murray, and Sendroy, (1925)). The reasons for the choice of 0.1 N HCl, with an assumed pH of 1.08 as the solution for determining E_0 in standardization of the cells, have been adequately discussed by Cullen, Keeler, and Robinson (1925).

Calculations.

The *CO_2 tension, p*, has been calculated from the CO_2 content of the air, with which the serum was equilibrated at atmospheric pressure, by the equation

$$p = \frac{\text{(per cent } CO_2 \text{ in gas)}}{100} \times (B - W) \tag{6}$$

H_2CO_3 concentrations were calculated by Equation 4, where B is the barometric pressure and W is the vapor tension of water.

$BHCO_3$ concentrations were calculated by subtracting $[H_2CO_3]$ from total CO_2 content of the serum. In no case was the pH sufficiently high to permit the presence of a significant amount of B_2CO_3.

pH was calculated from the observed electromotive force, E, as

$$\text{pH} = \frac{E - E_0}{0.06169} \tag{7}$$

The value of E_0 was determined from the E.M.F. observed with 0.1 N HCl in the hydrogen electrode vessel:

$$E_0 = \text{E.M.F.} - 1.08 \times 0.06169 \tag{8}$$

the pH of 0.1 N HCl being assumed to be 1.08.

pK′ was calculated by Equation 2 the values of $[H_2CO_3]$ and $[BHCO_3]$ having been estimated as above.

The results of our determinations are summarized in Table I.

Recalculation of pK_s' Values in the Literature.

In the literature of the past 6 years are several papers presenting carefully obtained data, from which the pK′ for serum at 38° either has been or may be calculated. In each case one or more of the constants involved in the calculation of results has been slightly or appreciably different from the constants employed in the present paper. In order to make the different pK_s' values comparable, therefore, we have recalculated them from the experimental data with the same factors, standards, and constants used above.

Warburg (1922).—At the end of Table XIII in Warburg's paper are data on twenty sera, of horse, ox, and man, at 38°. The values given are the mm. of CO_2 tension, the $[BHCO_3]$, and the pH determined with Bjerrum's standard, which, as pointed out on page 167 of Warburg's paper, gives pH values 0.048 higher than the Sörensen conductivity standard. The differences in the methods of calculating pH from observed electromotive force, E, depend upon the value taken for E_0 in the Sörensen (1912) equation.

$$\text{pH} = \frac{E - E_0}{0.0577 + 0.0002\ (t° - 18°)}$$

In our determinations, in which E_0 is determined with 0.1 N HCl, assumed to have pH of 1.08, the results approximate closely those of the Sörensen standard. We have therefore subtracted 0.048 from Warburg's pH values, in order to bring them to the same standard as ours. Exactly how precise this correction is we are not certain, but it appears to be within 0.01 pH. With the values of pH thus corrected, we have calculated pK_s' from Warburg's data by means of Equations 2 and 3, given above.[1]

[1] Warburg (1922) states that the difference between Bjerrum's and Sörensen's E_0 values for the 0.1 N calomel cell is 2.9 millivolts at room or body temperature. This means that pH calculated from Bjerrum's value would be 0.048 pH higher than that calculated from Sörensen's value. In a footnote on page 166 (1922) he states that the Bjerrum value at 38° is 331.3 millivolts. Sörensen's value for the 0.1 N calomel cell is 336.1 milli-

Cullen (1922).—Cullen published, from eleven analyses of horse plasma and six of horse serum, values for CO_2 contents, CO_2 tensions, and pH, at 38°. For standardizing his hydrogen electrodes Cullen used 0.1 N HCl, but assumed its pH to be 1.09, instead of 1.08. We have accordingly, for the present calculations, subtracted 0.01 from each of his pH values.

TABLE II.

pK′ Values Determined in Plasma or Serum by Different Authors.

Authors.	Material.	No. of determinations.	pK_a' with $\alpha = 0.510$.			pK_a' with $\alpha = 0.541$ (average).
			Minimum.	Maximum.	Average.	
Warburg (1922)....	Horse and ox serum.	20	6.07	6.13	6.10	6.126
Cullen (1922).....	Horse oxalate plasma.	11	6.086*	6.123	6.110*	6.136
"	Horse serum.	6	6.105*	6.119	6.113*	6.139
Cullen, Keeler, and Robinson (1925)..	Human "	15	6.043	6.095	6.064	6.090
Van Slyke, Hastings Murray, and Sendroy (1925)...	Horse "	13	6.09	6.15*	6.13*	6.156
Present writers....	Human "	16	6.094	6.134	6.105	6.131
Mean.....					6.104	6.130

* Excluding one determination, deviating rather widely from the rest of the series.

His determinations of the CO_2 content, in mM per liter, were carried out according to Van Slyke and Neill (1924). With Van Slyke and Sendroy's (1927) more exact factors now used in calculating the results of the Van Slyke-Neill analyses, the CO_2 concentrations in mM are 1.009 times as great as those calculated from the same analyses by the original factors given by Van Slyke and

volts at 38°. This would mean a difference of 4.8 millivolts corresponding to 0.078 pH. Since no original E.M.F. data are given, it is impossible to determine what value for the 0.1 N calomel cell he used in calculating pH, at 38°. In the absence of further information we have assumed that the value of 333.2 millivolts was used.

Neill, and used by Cullen. We have accordingly multiplied the CO_2 values of Cullen by 1.009 in order to make them comparable with our present data. The change effected by this correction is only to lower pK_s' by 0.004.

Cullen, Keeler, and Robinson (1925).—These authors have published data from analyses of fifteen human sera. They have used the same pH standard employed by ourselves. Their serum CO_2 contents were calculated by the original factors of Van Slyke and Neill, and we have corrected them with the factor 1.009, as above outlined.

In the calculations of $[H_2CO_3]$ from CO_2 tension Cullen, Keeler, and Robinson used the Bohr (1905) α of 0.541. The change to the 0.510 value, directly determined by Van Slyke, Sendroy, Hastings, and Neill (1928), lowers by 0.026 the pK_s' values calculated by Equation 5. The total effect of the two changes, required in order to calculate the data of Cullen, Keeler, and Robinson on the same basis of those of the present paper, is to lower the pK_s' values calculated from their determinations by 0.030 below the values computed by them from the same determinations.

Van Slyke, Hastings, Murray, and Sendroy (1925).—The pH standard was the same used in the present paper. The serum CO_2 contents given by these authors we have multiplied by 1.009 to change them to the values calculated by the new factors of Van Slyke and Sendroy (1927). The pK_s' values are then recalculated by means of Equation 5.

The pK_s' values calculated from the data of the above authors, together with our own, are summarized in Table II.

CONCLUSIONS.

The variations, due to causes which cannot be ascertained at present, are such that one does not appear justified in expressing the value of pK_s' to more than two decimal places. Consideration of the available data indicates that, when the solubility coefficient of CO_2 in serum is taken as 0.510, and the pH of 0.1 N HCl is taken as 1.08 in standardizing the electrometric pH determinations, the value of pK′ for serum at 38° is approximately *6.10*.

The pK_s' calculated from the same data with the Bohr α, 0.541, used by most previous authors, would be 6.13.

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XUM

STUDIES OF GAS AND ELECTROLYTE EQUILIBRIA IN BLOOD.

XIII. THE DISTRIBUTION OF CHLORIDE AND BICARBONATE IN THE BLOOD OF NORMAL AND PATHOLOGICAL HUMAN SUBJECTS.

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In 1923, Van Slyke, Wu, and McLean (1) published the results of a study of the factors responsible for the unequal distribution of hydrogen, chloride, and bicarbonate ions between erythrocytes and blood serum. This study was made upon blood brought into equilibrium, *in vitro*, with definite tensions of carbon dioxide and oxygen. Under these conditions it was found that the quantitative distribution of the diffusible ions could be approximately predicted by equations which were developed from the assumptions that osmotic pressure is equal in cells and serum, and that the distribution of diffusible ions between cells and serum is influenced by the non-diffusible ions according to the Gibbs-Donnan law. A later and more accurate experimental examination of the problem (2) emphasized the necessity of taking into account the factors which reduce the activity of ions in cells and serum and verified the fundamental correctness of the original postulates and equations. That the non-diffusible base bound by hemoglobin in the cells is the most important single constituent of blood in determining the distribution of chloride, bicarbonate, and hydrogen ions, seems established from the fact that any change, such as oxygenation or shift in reactions, which alters the amount of base bound by hemoglobin, brings about a predictable change in ionic distribution.

After the factors concerned with ionic distribution in horse blood brought artificially into equilibrium with O_2 and CO_2 had been

investigated, it seemed pertinent to study ionic distribution in blood freshly drawn from the human body.

This has been done with blood from normal individuals and from a series of hospital patients suffering from a variety of acute and chronic pathological conditions. As far as possible patients were chosen in whom there appeared to be the greatest probability of abnormal ionic distributions; *i.e.*, cardiac and pneumonia patients, in whom oxygenation may be deficient; nephritic patients, who may be anemic, have low plasma proteins, nitrogen retention, and acidosis; febrile patients with pneumonia and acute arthritis.

The following determinations have been made when adequate material was available: on the serum, electrometric pH, total CO_2, total base, chloride, water, and protein; on the cells, total CO_2, chloride, and water; on the whole blood, total oxygen content and capacity.

Our series consisted of nine observations on eight normal and thirty observations on twenty-nine pathological individuals. The latter were distributed as follows:

Disease.	No. of observations.
Pneumonia	9
Nephritis	13
Arthritis	5
Cardiac disease	3

Technique.

About 40 cc. of venous blood were drawn without stasis and defibrinated under oil. A sample of whole blood, about 6 cc. in amount, was transferred to a separate tube immediately for determination of its oxygen content and capacity. 0.1 cc. of 0.1 N KCN was added to the remainder for each 10 cc. of blood to inhibit respiration. The oil on the blood was replaced with paraffin, and the blood was centrifuged for 45 minutes at high speed. The bulk of the serum was then transferred to small sampling tubes over mercury. The last visible portion of the serum was removed by suction cautiously and slowly applied. The cells were then similarly transferred to sampling tubes over mercury, where they were kept for analysis. The CO_2 determinations were done immediately.

pH of serum was determined electrometrically at 38° in a Clark cell (3) as modified by Cullen (4). All values have been calculated

on the assumption that the pH of 0.1 N HCl is 1.080. No correction was attempted for diffusion potentials. Details of the method and of the calculation have been described in Paper VIII of this series (2).

Carbon dioxide and oxygen determinations were performed with the Van Slyke and Neill (5) manometric blood gas apparatus. The cell samples for CO_2 analysis were measured by weight. The sample was drawn into a 1 cc. stop-cock pipette, which was weighed. The sample of approximately 1 cc. was then delivered into the chamber of the Van Slyke-Neill apparatus, together with 6 cc. of N/60 lactic acid, which had previously been rendered CO_2-free by extraction in the apparatus. The pipette was then weighed again. The CO_2 values were calculated by the revised factors of Van Slyke and Sendroy (6).

Chlorides were determined in weighed samples of serum and cells by the method of Van Slyke (7). In the procedure the serum or cells are mixed in small Erlenmeyer flasks with 3 volumes of concentrated nitric acid containing a known amount of silver nitrate, and heated on a steam bath until the organic matter is destroyed and the chloride is precipitated as AgCl. The excess silver in solution is then titrated with sulfocyanate. A test of this method on sera and cells of various animal species, carried on in the laboratory of Professor D. Wright Wilson in Philadelphia and of the authors in New York (unpublished) has shown that in an occasional abnormal human serum, for a reason not yet identified, this method yields results 1 or 2 per cent too low. The method revealed no errors in cell analyses. In sera which gave low results, correct ones were obtained when the silver nitrate in water solution was mixed with the serum before nitric acid was added. This improvement, due to Professor Wilson, had not been introduced at the time the present work was done. The possible error of the original method is, however, not sufficient to be of significance in interpretation of our results.

Total base determinations were made by the modification of Fiske's benzidine sulfate method described on page 671 of the paper by Van Slyke, Hiller, and Berthelsen (8). Their gasometric micro method was not yet perfected at the time of the work here reported.

Serum proteins were estimated by gasometric micro-Kjeldahl determinations (9).

Values of water per gram or cc. were obtained by drying a known weight or volume to constant weight at 110°.

All the above determinations were done in duplicate, with good agreement. Consequently we believe that significant variations due to analytical error are excluded.

Calculations.

CO_2 tension of the blood was calculated from the total CO_2 content of the serum and the electrometrically determined pH, by Formula 5 of Table IV of Paper I of this series (10).

$$p = \frac{[CO_2]}{0.0591\ \alpha^0\ (10^{pH - pK'} + 1)}$$

The value for pK_s', from the accompanying paper (11) is taken as 6.10. The value of α^0, which indicates the cc. of CO_2 dissolved per gram of water in serum under 760 mm. CO_2 tension, is taken as 0.553 (12). Hence the equation becomes

$$p = \frac{[CO_2]}{0.0327\ (10^{pH_s - 6.10} + 1)}$$

mM $[H_2CO_3]_s$ per kilo of water in serum was calculated, from the above value of α^0, as

$$[H_2CO_3]_s = 0.0327\ p$$

mM $[H_2CO_3]_c$ per kilo of water in cells was calculated, from the value $\alpha^0 = 0.60$, found for cells (12), as

$$[H_2CO_3]_c = 0.0354\ p$$

Bicarbonate was estimated as the difference between total CO_2 and H_2CO_3.

$$[BHCO_3] = [CO_2] - [H_2CO_3]$$

The analyses were all performed on the partially oxygenated venous blood. In order to estimate the $[Cl]_c : [Cl]_s$ and $[BHCO_3]_c : [BHCO_3]_s$ ratios for a standard condition, for comparison of the results from different bloods, we have calculated in each case what the ratio would be if the blood were completely oxygenated. For this purpose we have used the empirical equation

$$c = 0.1\ (pH_s - 6.6)\ \frac{\text{reduced Hb}}{\text{total Hb}}$$

where c is the amount by which the determined ratios must be decreased to give the ratios of completely oxygenated blood. This empirical equation was deduced from results obtained with oxygenated and reduced horse blood in Paper VIII of this series (2). This c correction, although probably not strictly accurate for human blood, is in all cases so small that the error involved from basing it on horse blood data is negligible.

DISCUSSION.

The outstanding feature of the r_{Cl} and r_{HCO_3} values for human blood diagrammed in Fig. 1 is that in both normal and pathological cases the values at any given pH_s average definitely higher than those found for horse blood (2). To seek the factor which may cause this difference we inspect Equation 10 of Van Slyke, Wu, and McLean, in which they expressed a first approximation of the main factors affecting r, the ratio of the active concentration of Cl or HCO_3 anions in cells to the active concentration in serum.

$$r = 1 - \frac{[BP]_c + [Hb]_c - [BP]_s}{2\,([B]_s - [BP]_s)}$$

In the fraction on the right, which determines by how much r falls below unity, the two most important values at physiological pH ranges are $[BP]_c$, the base bound by cell colloids, and $[B]_s$, the total serum base. The latter is practically the same in horse blood as in human, about 170 to 175 milli-equivalents per kilo of serum water, or 156 to 160 milli-equivalents per liter of serum. But the $[BP]_c$ values appear to be appreciably different in the two species. Comparison of the data of Van Slyke, Wu, and McLean (1) on horse cell contents with those of Adair (13) on human cell contents indicates that the colloids of the latter bind less base at a given pH. In both cases (1, 13) the determinations were made of the base-binding power of the entire non-dialyzable constituents of the cells, which include, beside hemoglobin, other constituents, perhaps phosphatides, which bind less, but important, amounts of base. Thus Fig. 16 of our Paper VIII (2) indicates that at pH 7.4 recrystallized reduced horse hemoglobin binds per mol 1.7 equivalents of alkali, while Fig. 10 of Van Slyke, Wu, and McLean (1) indicates that at the same pH the dialyzed total cell contents bind 2.2 equivalents of alkali per mol of reduced hemoglobin.

The non-hemoglobin colloids of horse cells therefore appear to bind at pH 7.4 about 0.5 equivalent of base per mol of Hb. All such non-diffusible acids would, according to Van Slyke, Wu, and McLean, act alike in affecting the electrolyte distribution; hence all the base bound by non-diffusible cell constituents is represented by one symbol $[BP]_c$.

An estimate of the quantitative effect on the Cl and HCO_3 distribution ratios at normal blood pH_s which would be caused by the difference between $[BP]_c$ found in horse cell contents (1) and in human cell contents (13) may be made as follows.

At pH 7.2, which is about the cell pH of normal blood (1, 2) Fig. 10 of Van Slyke, Wu, and McLean (1) indicates that the non-diffusible constituents of oxygenated horse cells bind 2.16 equivalents of alkali per mol of Hb present. Table VII of Adair (13) indicates 1.23 as the corresponding value for human cell contents. If the hemoglobin content of the cells in each case is 30 millimols per kilo of water, the concentrations of $[BP]_c$ will be 64.8 and 36.9 milli-equivalents per kilo of water, respectively. From differentiation of Equation 10 of Van Slyke, Wu, and McLean (given above) we calculate that the change, Δr, in the distribution ratio, caused by a given change, $\Delta\ [BP]_c$, in $[BP]_c$, other values in the equation being constant, will be:

$$\Delta r = \frac{-\ \Delta\ [BP]_c}{2\ ([B]_s - [BP]_c)}$$

In both human and horse blood $[B]_s$ may be taken as 175 milli-equivalents, and $[BP]_s$ as 15, without significant error for our present calculation. The $\Delta[BP]_c$ caused by the change from horse to human blood is $36.9 - 64.8 = -27.9$ milli-equivalents. Hence

$$\Delta r = \frac{+\ 27.9}{320} = +\ 0.087$$

From the difference in base-binding power of the cell colloids we thus estimate that the r_{Cl} and r_{HCO_3} should be about 0.09 higher in human than in horse blood at the same pH.

Inspection of Fig. 1 shows that in fact the Cl and HCO_3 distribution ratios for both normal and pathological human blood lie above the curves found by Van Slyke, Hastings, Murray, and Sendroy (2) for horse blood, and by margins averaging not far from the above estimated 0.09. In the bloods from normal subjects

and patients with arthritis and cardiac disease there is an extreme variation of about ± 0.05 in either r_{Cl} or r_{HCO_3} at any given pH_s: such variation is to be expected from the possible variations in all

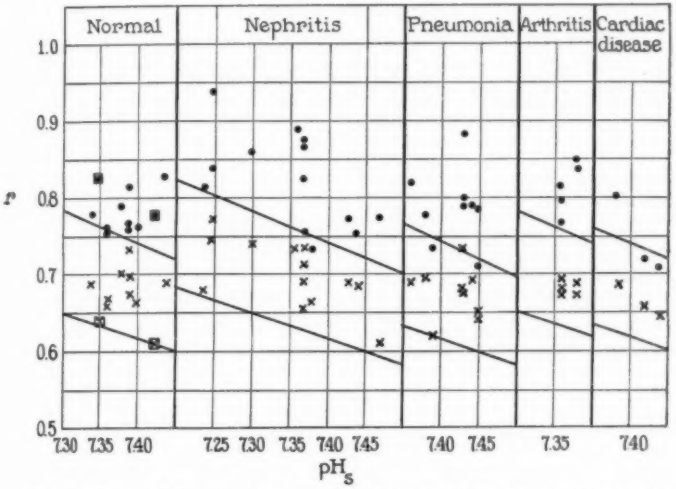

FIG. 1. Value of the chloride and bicarbonate distribution ratios in the oxygenated blood of normal and diseased human subjects.

● indicates $r_{HCO_3} = \dfrac{BHCO_3 \text{ per kilo } H_2O \text{ in cells}}{BHCO_3 \text{ per kilo } H_2O \text{ in serum}}$.

× indicates $r_{Cl} = \dfrac{Cl \text{ per kilo } H_2O \text{ in cells}}{Cl \text{ per kilo } H_2O \text{ in serum}}$.

⊡ and ⊠ are data of Dill, van Caulaert, Hurxthal, Stoddard, Bock, and Henderson (14) on normal men.

The solid curves represent the mean values for the two ratios obtained in oxygenated *horse* blood by Van Slyke, Hastings, Murray, and Sendroy (2). The upper curve in the diagram for each group represents r_{Cl}, the lower r_{HCO_3}.

the factors that influence r. At any given pH_s there is no marked difference between normal and pathological bloods in the r_{Cl} and r_{HCO_3} values; in the nephritis and pneumonia blood the variation at given pH_s is somewhat greater, about ± 0.07, and in addition the total range of r values is further extended by the greater

range in pH_s.[1] That the r variation at given pH_s is not more markedly greater than in normal men may be attributed to a fair degree of constancy in the two single factors, *the hemoglobin concentration in the cells* and the *total base of the serum*, which are most important in fixing the value of r.[2]

It is of interest that the relation of r_{Cl} to r_{HCO_3} is the same in the blood of normal and pathological individuals. As the average of our determinations on human blood, we find

$$\frac{[Cl]_c}{[Cl]_s} : \frac{[BHCO_3]_c}{[BHCO_3]_s} = 0.87$$

In horse blood Van Slyke, Hastings, Murray, and Sendroy (2) found the ratio of r_{Cl} to r_{HCO_3} to be 0.81. The difference between chloride and bicarbonate distributions is presumably attributable to factors which affect the activity coefficients of Cl and HCO_3 anions in the cells, and which we have thus far been unable to identify.

CONCLUSIONS.

The distribution of chloride and bicarbonate between cells and serum has been studied in human venous blood. The distribution ratios [cell Cl] : [serum Cl] and [cell $BHCO_3$] : [serum $BHCO_3$] have been found to average several per cent higher than in horse blood at the same pH_s. The difference approximates that calculated by Equation 10 of Van Slyke, Wu, and McLean (1) from the lesser base-binding power of the colloid constituents of human cells, indicated by the analyses of Adair (13).

Studies of blood from patients with nephritis (severe but not uremic), cardiac disease, pneumonia, and acute arthritis, representing a fairly wide range of pathological conditions, failed to reveal evidence that in any of these conditions the mechanism

[1] Henderson, Bock, Dill, Hurxthal, and van Caulaert (15) have recently published analyses of blood from two nephritic patients in extremis. The pH_s values were much more depressed, to the neighborhood of 7.00, than in any of our cases, and the r values correspondingly higher, r_{Cl} being 0.92 in venous blood, 0.90 in arterial, while r_{HCO_3} exceeded unity.

[2] The much more numerous analyses of blood in various pathological conditions by Peters and his collaborators (16) indicate that even in disease it is rather rare for the serum total base or the ratio (O_2 capacity): (cell volume) to deviate by more than 10 per cent from the normal range.

controlling the electrolyte distribution becomes qualitatively altered, or that very marked quantitative deviations from the normal distribution ratios occur, other than the predicted r changes due to blood pH variation. The only definite quantitative deviation from the normal was a tendency for r_{HCO_3} values to be somewhat higher in part of the nephritic bloods.

The failure of the r_{Cl} and r_{HCO_3} ratios to deviate in the pathological bloods more markedly from the ratios observed at the same pH_s in normal blood appears attributable to the facts that the two factors which are theoretically most important in determining the values of the ratios at any given pH are the total base concentration in the serum and the hemoglobin concentration in the cells, and that neither of these is liable to great fluctuations in the conditions studied.

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TABLE I.

Normal Subjects.

Subject.	Material.	H_2O content.		pH_s		$[CO_2]$	CO_2 tension.	$[H_2CO_3]$	$[BHCO_3]$	[Cl]	HbO_2 content.	HbO_2 capacity.	Oxygenation of Hb.	Total base.	$\frac{[BHCO_3]_c}{[BHCO_3]_s}$.		$\frac{[Cl]_c}{[Cl]_s}$.	
				Electrometric.	Colorimetric.										Found.	Calculated to 100 per cent oxygenation.	Found.	Calculated to 100 per cent oxygenation.
		gm. per cc.	*gm. per gm.*			*mM per kg. H_2O*	*mm.*	*mM per kg. H_2O*	*mM per kg. H_2O*	*m.-eq. per kg. H_2O*	*mM per l. blood*	*mM per l. blood*	*per cent*	*m.-eq. per kg. H_2O*				
A. B. H.	Serum.	0.938	0.912	7.38	7.41	32.95	50.2	1.64	31.31	108.4					0.829	0.790	0.738	0.699
	Cells.		0.691			27.75	50.2	1.78	25.97	80.1	4.66	9.22	50.6					
J. M.	Serum.	0.934	0.909	7.36	7.38	32.67	52.1	1.70	30.97	110.8					0.811	0.763	0.716	0.668
	Cells.		0.670			26.94	52.1	1.84	25.10	79.3	3.63	9.88	36.8					
W. N.	Serum.	0.933	0.919	7.39	7.46	31.09	47.8	1.56	29.53	108.9					0.798	0.768	0.727	0.697
	Cells.		0.678			25.26	47.8	1.69	23.57	79.2	5.66	8.95	63.2					
G.	Serum.	0.931	0.915	7.44		28.54	38.1	1.25	27.29	111.5				173.4	0.852	0.828	0.711	0.687
	Cells.		0.685			24.60	38.1	1.35	23.25	79.3	6.41	8.92	71.8					
C. D.	Serum.	0.934		7.40		33.67	49.0	1.60	32.07	109.1				170.0	0.866	0.812	0.768	0.714
	Cells.		0.690			29.52	49.0	1.73	27.79	83.8	3.27	9.99	32.8					
L. L.	Serum.		0.911	7.39		34.24	51.1	1.67	32.57	104.3				178.5	0.862	0.815	0.775	0.728
	Cells.		0.668			29.84	51.1	1.81	28.03	80.8	3.93	9.25	42.5					

G. S.	Serum.	0.917	7.34	35.55	59.1	1.93	33.62	106.2					0.828	0.781	0.731	0.684
	Cells.	0.680		29.81	59.1	2.09	27.82	77.6	4.05	10.95	37.0					
W. N.	Serum.	0.916	7.39	32.57	48.6	1.59	30.98	107.1				175.6	0.797	0.758	0.712	0.673
	Cells.	0.683		26.40	48.6	1.72	24.68	76.3	4.81	9.36	51.4					
F.	Serum.	0.913	7.36	34.00	54.1	1.77	32.23	104.4					0.801	0.758	0.700	0.657
	Cells.	0.685		27.73	54.1	1.91	25.82	73.1	4.25	9.68	43.9					
Maximum			7.44											0.828		0.728
Minimum			7.36											0.758		0.657
Mean			7.38											0.786		0.689

TABLE II.

Nephritic Subjects.

Subject.	Material.	H_2O content.		pH_s		$[CO_2]$	CO_2 tension.	$[H_2CO_3]$	$[BHCO_3]$	[Cl]	HbO_2 content.	HbO_2 capacity.	Oxygenation of Hb.	Total base.	Serum proteins.	$\frac{[BHCO_3]_c}{[BHCO_3]_s}$		$\frac{[Cl]_c}{[Cl]_s}$	
		Electrometric.	Colorimetric.													Found.	Calculated to 100 per cent oxygenation.	Found.	Calculated to 100 per cent oxygenation.
		gm. per cc.	*gm. per gm.*			*mM per kg. H_2O*	*mm.*	*mM per kg. H_2O*	*mM per kg. H_2O*	*m.-eq. per kg. H_2O*	*mM per l. blood*	*mM per l. blood*	*per cent*	*m.-eq. per kg. H_2O*	*gm. per kg. H_2O*				
Simc.	Serum.	0.923		7.25		15.55	31.5	1.03	14.52	110.9				171.7	63.7	0.953	0.939	0.784	0.770
	Cells.		0.688			14.94		1.12	13.82	86.9	3.18	4.08	78.0						
Jacks.	Serum.	0.922		7.24		18.41	38.0	1.24	17.17	101.4				163.6	66.7	0.829	0.815	0.694	0.680
	Cells.		0.683			15.56		1.35	14.21	70.4	3.38	4.29	78.8						
Giaq.	Serum.	0.943		7.43		29.27	39.9	1.30	27.97	107.1				166.5		0.828	0.773	0.745	0.690
	Cells.		0.672			24.55		1.41	23.14	79.8	3.34	6.11	54.7						
Cunn.	Serum.	0.928		7.37		29.49	46.0	1.50	27.99	110.7				166.8	80.3	0.772	0.756	0.671	0.655
	Cells.		0.672			23.25		1.63	21.62	74.3	6.59	8.37	78.8						
Carr.	Serum.	0.935		7.47		28.44	35.3	1.15	27.29	106.8				162.6	43.6	0.776	0.774	0.612	0.610
	Cells.		0.682			22.40		1.25	21.15	65.5	5.46	5.58	97.8						
Nab.	Serum.	0.947		7.38		27.46	41.8	1.37	26.09	114.3				165.5		0.762	0.732	0.690	0.660
	Cells.		0.671			21.36		1.48	19.88	78.8	3.18	5.22	61.0						

Nab.	Serum.	0.947		7.38		27.46	41.8	1.37	26.09	114.3				165.5		0.762	0.732	0.690	0.660
	Cells.		0.671			21.36		1.48	19.88	78.8	3.18	5.22	61.0						
Ols.	Serum.	0.941		7.36		23.65	37.7	1.23	22.42	109.5				161.7	58.6	0.843	0.825	0.707	0.689
	Cells.		0.685			20.26		1.34	18.92	77.4	4.44	5.80	76.5						
Cor.	Serum.	0.946		7.44		34.62	46.2	1.51	33.11	108.0				159.3	55.9	0.806	0.756	0.736	0.686
	Cells.		0.713			28.33		1.64	26.69	79.5	3.13	7.64	41.0						
Cunn.	Serum.	0.937		7.36		29.55	47.0	1.54	28.01	109.8						0.920	0.888	0.765	0.733
	Cells.		0.641			27.46		1.66	25.80	84.0	4.46	7.62	58.5						
Val.	Serum.	0.948		7.37		29.83	46.6	1.52	28.31	112.1						0.930	0.875	0.768	0.713
	Cells.		0.689			27.97		1.65	26.32	86.1	2.39	8.34	28.7						
Clar.	Serum.	0.933			7.37	31.90	49.8	1.63	30.27	101.4						0.906	0.871	0.768	0.733
	Cells.		0.703			29.20		1.76	27.44	77.8	4.88	8.92	54.7						
Wohl.	Serum.	0.932		7.30		23.18	41.9	1.37	21.81	89.4				145.0	58.1	0.911	0.861	0.791	0.741
	Cells.		0.696			21.39		1.52	19.87	70.8	1.60	5.60	28.6						
McK.	Serum.	0.935		7.25		15.86	32.1	1.05	14.81	97.8				144.5	57.1	0.847	0.839	0.754	0.746
	Cells.		0.699			13.70		1.14	12.56	73.7	4.05	4.63	87.4						
Maximum				7.44													0.939		0.770
Minimum				7.25													0.732		0.610
Mean				7.35													0.824		0.700

Case Cunn. was the arteriosclerotic type of Bright's disease; Cor. was an acute hemorrhagic case which subsequently recovered. The other cases were of chronic hemorrhagic Bright's disease (Addis' classification) in advanced stages with marked functional impairment, but none was in actual uremia.

TABLE III.

Pneumonia.

Subject.	Material.	H_2O content.		pH_s		$[CO_2]$	CO_2 tension.	$[H_2CO_3]$	$[BHCO_3]$	[Cl]	HbO_2 content.	HbO_2 capacity.	Oxygenation of Hb.	$\frac{[BHCO_3]_c}{[BHCO_3]_s}$.		$\frac{[Cl]_c}{[Cl]_s}$.	
				Electrometric.	Colorimetric.									Found.	Calculated to 100 per cent oxygenation.	Found.	Calculated to 100 per cent oxygenation.
		gm. per cc.	*gm. per gm.*			*mM per kg. H_2O*	*mm.*	*mM per kg. H_2O*	*mM per kg. H_2O*	*m.-eq. per kg. H_2O*	*mM per l. blood*	*mM per l. blood*	*per cent*				
Tay.	Serum.		0.919	7.38	7.39	23.46	35.7	1.17	22.29	95.7				0.801	0.777	0.718	0.694
	Cells.		0.694			19.12	35.7	1.26	17.86	68.7	6.34	8.96	70.8				
Sa.	Serum.	0.929	0.913	7.44		35.98	48.0	1.57	34.41	104.5				0.847	0.791	0.747	0.691
	Cells.		0.693			30.84	48.0	1.70	29.14	78.1	2.84	8.58	33.1				
Pon.	Serum.	0.935		7.43		31.00	42.3	1.38	29.62	106.2				0.835	0.790	0.717	0.682
	Cells.		0.690			25.90	42.3	1.50	24.40	76.2	5.21	9.02	57.8				
Ye.	Serum.	0.925		7.43		31.64	43.2	1.41	30.23	106.7				0.812	0.798	0.690	0.676
	Cells.		0.693			25.99	43.2	1.53	24.46	73.6	7.26	8.76	82.9				
Eis.	Serum.	0.931		7.45		31.00	40.5	1.32	29.68	101.0				0.834	0.784	0.690	0.640
	Cells.		0.686			26.18	40.5	1.43	24.75	69.7	3.16	7.57	41.7				
Kun.	Serum.	0.924			7.43	30.72	41.9	1.37	29.35	111.4				0.903	0.887	0.748	0.732
	Cells.		0.604			27.98	41.9	1.48	26.50	83.3	6.53	8.27	79.0				

Kuh.	Serum.	0.924			7.43	30.72	41.9	1.37	29.35	111.4				0.903	0.887	0.748	0.732
	Cells.		0.604			27.98	41.9	1.48	26.50	83.3	6.53	8.27	79.0				
Ma.	Serum.	0.923		7.36	7.36	33.59	52.9	1.73	31.86	102.2				0.862	0.820	0.734	0.690
	Cells.		0.686			29.21	52.9	1.87	27.34	75.0	4.34	10.33	42.0				
Cot.	Serum.	0.942		7.45	7.44	34.71	45.4	1.48	33.23	91.7				0.750	0.710	0.688	0.648
	Cells.		0.683			26.53	45.4	1.61	24.92	63.1	4.33	8.16	53.0				
Hir.	Serum.	0.934			7.38	31.62	48.1	1.57	30.05	111.2				0.771	0.734	0.657	0.620
	Cells.		0.683			24.86	48.1	1.70	23.16	73.2	4.40	8.47	52.0				
Maximum															0.887		0.732
Minimum															0.710		0.620
Mean															0.788		0.674

TABLE IV.

Acute Arthritis and Cardiac Disease.

Subject.	Material.	H_2O content.	pH_s electrometric.	$[CO_2]$	CO_2 tension.	$[H_2CO_3]$	$[BHCO_3]$	[Cl]	HbO_2 content.	HbO_2 capacity.	Oxygenation of Hb.	$\frac{[BHCO_3]_c}{[BHCO_3]_s}$.		$\frac{[Cl]_c}{[Cl]_s}$.	
												Found.	Calculated to 100 per cent oxygenation.	Found.	Calculated to 100 per cent oxygenation.
Arthritis.															
		gm. per cc.	*gm. per gm.*		*mM per kg. H_2O*	*mm.*	*mM per kg. H_2O*	*mM per kg. H_2O*	*m.-eq. per kg. H_2O*	*mM per l. blood*	*mM per l. blood*	*per cent*				
Dob.	Serum.	0.928		7.36	34.46	54.9	1.79	32.67	105.2				0.810	0.764	0.734	0.680
	Cells.		0.664		28.40	54.9	1.94	26.46	77.2	3.05	7.76	39.3				
Stil.	Serum.	0.932		7.36	33.25	52.9	1.73	31.52	108.4				0.838	0.795	0.732	0.689
	Cells.		0.704		28.27	52.9	1.87	26.40	79.3	3.52	8.10	43.5				
Lin.	Serum.	0.921		7.38	29.20	44.4	1.45	27.75	109.7				0.887	0.836	0.734	0.683
	Cells.		0.702		26.18	44.4	1.57	24.61	80.6	2.88	8.04	35.9				
Coh.	Serum.	0.931		7.36	30.78	49.1	1.61	29.17	109.2				0.870	0.816	0.729	0.675
	Cells.		0.712		27.10	49.1	1.74	25.36	79.6	2.54	8.80	28.9				
Wee.	Serum.	0.932		7.38	35.53	54.0	1.76	38.77	106.2				0.896	0.849	0.717	0.670
	Cells.		0.681		32.17	54.0	1.91	30.26	76.1	3.59	9.13	39.4				
Maximum														0.849		0.689
Minimum														0.769		0.670
Mean														0.812		0.640

Maximum														0.849		0.689
Minimum														0.769		0.670
Mean														0.812		0.640
							Cardiac disease.									
Gebr.	Serum.	0.937		7.44	34.00	45.4	1.48	32.52	106.9				0.735	(0.707)	0.673	(0.644)
	Cells.		0.714		25.53	45.4	1.61	23.92	71.9							
Caff.	Serum.	0.925		7.42	33.22	46.4	1.52	31.70	107.8				0.762	0.738	0.681	0.657
	Cells.		0.688		25.78	46.4	1.64	24.14	73.4	6.42	9.02	71.2				
Schw.	Serum.	0.927		7.38	33.65	51.2	1.67	31.98	108.0				0.835	0.800	0.710	0.685
	Cells.		0.700		28.52	51.2	1.81	26.71	76.7	4.61	10.00	46.1				

THE OXIDATION OF DIXANTHYDRYL UREA, A MICRO METHOD FOR DETERMINING UREA.

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The method described by Kiech and Luck (1) for the estimation of urea suffers from three undesirable features. Like many gravimetric methods it requires special apparatus and the expenditure of much time. A fairly large amount of urea, somewhat more than 0.5 mg., is also needed. This quantity is contained in about 20 cc. of the tungstic acid filtrate from normal blood and tissues and only too often it was found inconvenient to spare so large a quantity. We were therefore driven to exercise further economies in time and material.

In the course of his extensive description of xanthydrol, Fosse (2) pointed out that this substance is readily oxidized to xanthone. From this it occurred to us to investigate the action of oxidizing agents upon dixanthydryl urea in the hope of developing a quantitative volumetric method for the estimation of urea. A few preliminary experiments showed that dixanthydryl urea dissolved in strong acids to give fluorescent solutions of a brilliant canary-yellow color, which on oxidation with potassium permanganate, became colorless. As the end-point could be determined quite readily under the optimum conditions, we were enabled to utilize these facts as the basis of a quantitative method. Before describing the procedure, it would be well to point out that oxidation appears to proceed far beyond the formation of xanthone.

Method.

2 to 5 cc. of the tungstic acid filtrate (1, 3) contained in a 15 cc. centrifuge tube are diluted to 5 cc. with water.[1] (For normal

[1] We have been employing Eastman's preparation of xanthydrol. We have not succeeded in obtaining complete solution of the substance in methyl alcohol, although Fosse appears to have done so. The solution

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blood use 5 cc. of filtrate.) 5 cc. of glacial acetic acid and 0.5 cc. of 10 per cent xanthydrol in methyl alcohol[2] are added. The contents must be intimately mixed. After 1 hour the tube is centrifuged, the supernatant fluid is decanted off, and the precipitate of dixanthydryl urea washed once with 5 cc. of a saturated solution of dixanthydryl urea in methyl alcohol. This serves to remove the excess xanthydrol. The tube is again centrifuged and the fluid removed by decantation. The excess of alcohol which clings to the precipitate and the wall of the tube is removed by drying. A few minutes at 100° is sufficient for this purpose. 8 cc. of cold 1:1 sulfuric acid (equal volumes of water and concentrated acid) are now added.[3] The residue dissolves slowly to form a yellow solution which is washed with 32 cc. of water into an evaporating basin of white porcelain, 10 cm. in diameter. The contents are heated to 70–75° and titrated at this temperature with 0.05 N potassium permanganate contained in a 5 cc. burette. It is advisable to carry out the titration in a bright, white light having at hand a second porcelain basin containing 40 cc. of water. The latter is of great assistance as a standard and is used for purposes of comparison as the end-point is neared. It should not be artificially illuminated. If the basin containing the solution of dixanthydryl urea be removed from beneath the burette and placed beside the standard, or between two such standards after each addition of permanganate the end-point may readily be determined. The fluid must be titrated until the last tint of yellow has disappeared.

It is necessary to maintain an acid concentration of 10 per cent (by volume). Since the latter changes by dilution during the titration, it is advisable to add, when near the end-point, an addi-

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used in this work was prepared by suspending 10 gm. of xanthydrol in 90 gm. of pure methyl alcohol, shaking at intervals during 3 days, and filtering.

[2] Since this method is applicable to the estimation of dixanthydryl urea however prepared, it is apparent that the use of other protein precipitants or conditions of precipitations are permissible,—provided only that the subsequent formation of dixanthydryl urea be quantitative and productive of a pure, crystalline ureide.

[3] Complete solution of the dixanthydryl urea is essential. It is advisable to pulverize the precipitate by grinding it against the bottom of the centrifuge tube with a stirring rod before adding the sulfuric acid.

tional amount of the 1:1 sulfuric acid equal to one-fourth of the volume of permanganate employed.

Not more than 3 cc. of permanganate should be employed in the titration. Somewhat beyond this point the relationship between the amounts of permanganate and urea ceases to be linear. Below this upper limit 1 cc. of 0.05 N $KMnO_4$ = 0.093 mg. of urea.

For the estimation of urea in urine, dilute 1 cc. of urine in a volumetric flask to 100 cc. Transfer 1 cc. of the diluted urine to a 15 cc. centrifuge tube, add 4 cc. of water, 5 cc. of glacial acetic acid, and 0.5 cc. of the methyl alcohol solution of xanthydrol. Continue with the procedure given above.

EXPERIMENTAL.

Preparation of Dixanthydryl Urea.

To 40 cc. of 0.20 per cent urea were added 140 cc. of glacial acetic acid and 20 cc. of 10 per cent xanthydrol in methyl alcohol. The latter was added in 5 cc. portions at 5 minute intervals. 1 hour and 25 minutes after the last addition, the mixture was centrifuged and the crystalline precipitate washed with 100 cc. of ethyl alcohol and again centrifuged. The residue was washed onto a suction filter with 50 cc. of ethyl alcohol and dried at 60°. The product was employed in the first few experiments and for preparation of the saturated solution of dixanthydryl urea in methyl alcohol.

Optimum Concentration of Sulfuric Acid.

33.3 mg. of dixanthydryl urea were dissolved in 100 cc. of 1:1 sulfuric acid. 3 cc. portions were diluted with water and sulfuric acid to volumes of 40 cc. and sulfuric acid concentrations of 5 to 50 per cent. They were titrated at 60–70° with 0.059 N potassium permanganate. It was found that the titration value is dependent upon the concentration of sulfuric acid and is indeed a linear function of the concentration between 5 and 30 per cent. For future work it was decided to employ an acid concentration of 10 per cent. The color given by the dixanthydryl urea in 5 per cent sulfuric acid was relatively faint. It is also obvious that a

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maximum depth of color with a maximum titration value is desirable. Nevertheless it was not permissible to use very high concentrations of sulfuric acid. In an acid concentration of 50 per cent the fluid became brownish during titration, the end-point was very obscure and, indeed, charring appeared to have proceeded. In order to minimize the possibility of such a happening, the relatively low acid concentration of 10 per cent was selected. This was used in all later work. Only on completion of the experiments described in this paper was it discovered that in view of the approximately linear relationship between acid concentration and cc. of permanganate the use of at least 20 per cent sulfuric acid would probably have been permissible. As the titrations have proceeded satisfactorily under the conditions described we have not seen fit to attempt this change, although the delicacy of the method might be thereby enhanced.

Volume of Sulfuric Acid.

Although the quantity of permanganate required for the oxidation of a given amount of dixanthydryl urea is a function of the concentration of sulfuric acid it is independent of the volume.

Five portions of dixanthydryl urea solution, each of 3 cc., were each diluted with 12 cc. of water. Increasing amounts of 10 per cent sulfuric acid (15 cc., 25 cc., 25 cc., 40 cc., 40 cc.) were added. The solutions were then titrated at 70–75° with 0.059 N $KMnO_4$. The following titration values were obtained: 1.34 cc., 1.29 cc., 1.32 cc., 1.28 cc., and 1.33 cc. respectively.

The experiment was repeated with 4 cc. portions of dixanthydryl urea solution to which were added 16 cc. of water and 5 to 40 cc. of 10 per cent sulfuric acid. Titrations were made with 0.0501 N $KMnO_4$ at 70–75°. Fourteen samples were run. All values fall between 2.05 and 2.19 cc. of potassium permanganate.

Oxidation of Varying Quantities of Dixanthydryl Urea.

66.6 mg. of dixanthydryl urea were dissolved in 1:1 sulfuric acid and made up to a volume of 200 cc. Ten portions of increasing size (2 cc. to 8 cc.) were diluted with water and sulfuric acid to final volumes of 40 cc. and acid concentration of 10 per cent. Titrations were made at 70–75°.

It was found that up to 2 mg. of dixanthydryl urea the quantity

of potassium permanganate required for the oxidation is a linear function of the amount of ureide present. All values for the urea equivalent (in mg.) of 1.0 cc. of 0.05 N potassium permanganate fell between 0.091 and 0.096. The average was 0.093.

Application of Method to Solutions of Urea.

An aqueous solution of urea containing 100 mg. of urea per liter was prepared. Portions of the solutions contained in small

TABLE I.
Estimation of Urea.

Urea solution.	0.0501 N $KMnO_4$.	Urea found.	Urea calculated.
cc.	*cc.*	*mg.*	*mg.*
1.0	1.04	0.099	0.100
1.0	1.10		
1.5	1.66	0.150	0.150
1.5	1.57		
2.0	2.06	0.198	0.200
2.0	2.19		
2.5	2.70	0.247	0.250
2.5	2.62		
3.0	3.30	0.305	0.300
3.0	3.26		
3.5	3.32	0.313	0.350
3.5	3.39		
4.0	3.96	0.351	0.400
4.0	3.58		

centrifuge tubes were made up to 5 cc. with water. The urea was precipitated as dixanthydryl urea by the addition of 5 cc. of glacial acetic acid and 0.5 cc. of xanthydrol solution. The washed precipitates were estimated by oxidation with potassium permanganate, according to the procedure already described. The results are presented in Table I.

Estimation of Urea in Blood.

Six portions of rabbit blood, each of 2 cc., were treated as follows: To the first was added 14 cc. of water, to the second 13 cc. of water and 1 cc. of urea solution (0.25 mg. per cc.), to the third 12 cc. of water and 2 cc. of urea, to the fourth 11 cc. of water and 3 cc. of urea, to the fifth 10 cc. of water and 4 cc. of urea, and to the sixth 8 cc. of water and 6 cc. of urea. Each received 2 cc. of sodium tungstate (10 per cent) and 2 cc. of sulfuric acid (0.66 N). Of the six filtrates which were then obtained, 2 and 3 cc. portions of each were treated with water, glacial acetic acid, and xanthydrol according to the routine procedure.

The experiment was repeated upon the blood of another rabbit. The results of both experiments are summarized in Table II.

TABLE II.

Recovery of Urea from Blood.

Urea in 2 cc. of blood.

Experiment 1.		Experiment 2.	
Found.	Calculated.	Found.	Calculated.
mg.	*mg.*	*mg.*	*mg.*
1.00		0.50	
1.29	1.25	0.74	0.75
1.55	1.50	1.06	1.00
1.82	1.75	1.28	1.25
2.06	2.00	1.56	1.50
2.47	2.50	2.03	2.00

Each value represents the average of two determinations, one of which was obtained from 2 cc. of filtrate, and the other from 3 cc.

Estimation of Urea in Animal Tissue.

An adult female rat was killed by stunning and deeply incising the thorax. The blood was collected over potassium oxalate and the liver and muscles of one hind limb excised and frozen with liquid air. The powdered tissues, 4 gm. samples, were treated with tungstic acid as described in a preceding paper (1). Urea estimations were made upon portions of each filtrate by the volumetric method described in this paper. These results, together with those from a second rat, are presented in Table III.

Use of Tanret's Reagent as a Protein Precipitant.

Kiech and Luck (1) reported that the application of their method to muscle gave impure, yellowish precipitates of the ureide. In the course of the experiments which have been summarized in Table III, trouble was again experienced with the muscle preparations. The unwashed dixanthydryl urea obtained after the first centrifugation was somewhat discolored. Practically all of the colored impurity appeared to dissolve in the methyl alcohol for the washed product was quite white. Nevertheless when the attempt to dissolve the dried precipitates in 1:1 sulfuric acid was made, there appeared to be a small portion of the muscle samples which resisted solution.

TABLE III.

Urea Content of Rat Blood, Liver, and Muscle.

Tissue.	Volume of filtrate used.		0.05 N $KMnO_4$.		Urea in 100 gm. of tissue.	
	Rat 1.	Rat 2.	Rat 1.	Rat 2.	Rat 1.	Rat 2.
	cc.	cc.	cc.	cc.	mg.	mg.
Blood	2	3	0.41	0.64	19.1	19.8
"	3	5	0.64	0.99	19.8	18.4
Liver	2	5	0.38	1.00	17.7	18.6
"	3	5	0.60	0.99	18.6	18.4
Muscle	2	5	0.28	0.94	13.0	17.5
"	3	5	0.49	0.94	15.2	17.5

It seemed advisable therefore to check the tungstate procedure by the use of another protein precipitant. For this purpose we employed Tanret's reagent[4] as modified by Fosse, Robyn, and François (4).

Duplicate 4 gm. samples of frozen, powdered liver and muscle were weighed out. Two rats had to be employed to supply enough liver tissue, the latter being intimately mixed before weighing. The first of each pair of tissue samples was treated with 28 cc. of ice-cold water, 4 cc. of ice-cold sodium tungstate (10 per cent), and 4 cc. of ice-cold sulfuric acid (0.66 N). The second sample of each pair received 32 cc. of water, and 4 cc. of Tanret's

[4] Tanret's reagent consists of: mercuric chloride 2.71 gm., potassium iodide 7.20 gm., and glacial acetic acid 66.6 cc.; diluted with water to 100 cc.

reagent. Of the four filtrates which were then obtained, 3 cc. and 5 cc. portions of each were treated with glacial acetic acid and xanthydrol. The rest of the procedure was that described in the first portion of this paper.

This experiment was performed a second and third time. In a fourth experiment a pregnant female rat (18th day of gestation) was employed and analyses were made upon the fetus as well. This animal had received urea by subcutaneous injection 25 minutes before. The results are summarized in Table IV.

TABLE IV.

Tanret's Reagent as Protein Precipitant.

Tissue.	Protein precipitant.	Volume of filtrate used.	Urea content per 100 gm. of tissue.			
			Experiment 1.	Experiment 2.	Experiment 3.	Experiment 4.
		cc.	*mg.*	*mg.*	*mg.*	*mg.*
Liver.....	Tungstic acid.	3	17.5	20.6	27.6	83.7
"	" "	5	16.5	19.2	25.6	
"	Tanret's reagent.	3	16.9	21.5	27.0	80.5
"	" "	5	17.5	21.2	25.9	
Muscle.....	Tungstic acid.	3	17.6	18.8		54.5
"	" "	5	16.6	17.3	24.8	
"	Tanret's reagent.	3	22.2	21.2	26.3	88.3
"	" "	5	23.3	21.0	26.3	
Whole fetus.....	" "	5				54.3
" "	" "	5				58.7

In Experiment 4, the animal received urea by subcutaneous injection 25 minutes before being killed.

From these it may be concluded that tungstic acid and Tanret's reagent are equally satisfactory for the precipitation of the proteins of the liver and fetus. Identical urea values are obtained.

When applied to muscle, however, tungstic acid permits the passage into the filtrate of some product which contaminates the dixanthydryl urea ultimately obtained, interferes with its solution in 1:1 sulfuric acid, and leads to low titration values.

Believing that it might be possible to precipitate the interfering substance by adding Tanret's reagent to tungstic acid filtrates of muscle, we made several experiments with that end in view. The addition of 0.1 to 0.3 cc. of Tanret's reagent gave no precipi-

tate. It was found that the subsequent addition of glacial acetic acid and xanthydrol gave precipitates of dixanthydryl urea which were less contaminated, and which dissolved more readily in 1:1 sulfuric acid, but complete solution of the precipitates was not obtained. The permanganate titration values were still inclined to be lower that those which followed the use of Tanret's reagent only.

Were it not for the need of estimating substances other than urea in the protein-free tissue extracts we would be inclined to use Tanret's reagent as the protein precipitant. We have however been mindful of the value of using a reagent which would be of general utility in tissue analysis. Thus we have found Tanret's reagent quite unsuitable if the estimation of amino acids be desired. It is nevertheless necessary to use this reagent as the protein precipitant in estimating the urea content of muscle.

The presence of glycogen in the tissue extracts does not interfere in the determination of urea by this method.

SUMMARY.

Dixanthydryl urea dissolves in sulfuric acid to give fluorescent yellow solutions which are rendered colorless by oxidation with potassium permanganate. These facts have been employed in the development of a volumetric method for the estimation of urea. 0.1 mg. may be estimated with an experimental error of about 5 per cent.

Alan Archer McCray and Frank Hilton Smith assisted in part of this work.

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Addendum.—Since this work was submitted for publication, a paper by F. Beattie appeared (*Biochem. J.*, 1928, xxii, 711) in which dixanthydryl urea is estimated colorimetrically by comparison of the yellow solution, here described, against a standard.

A STUDY OF THE ESTIMATION OF CHLORIDE IN BLOOD AND SERUM.

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Many modifications have been suggested for the determination of chloride in blood and other body fluids. The simplest and most thoroughly studied is a method proposed by Van Slyke (1) involving ashing of serum with concentrated nitric acid in the presence of silver nitrate to prevent volatilization of the chloride.

In making a preliminary study of Van Slyke's method before using it for a series of analyses, we attempted to cut down the period of digestion (2 hours on a water bath) by boiling the mixture gently over a micro burner until the solution was clear. When this was done it was noted that the results tended to be higher than those obtained by the regular Van Slyke procedure. The silver nitrate in nitric acid used by Van Slyke forms an immediate voluminous precipitate, protein and silver chloride, when added to serum. In order to diminish the possibility of occlusion of chloride or silver, it was thought that the reagents might better be added separately. Aqueous silver nitrate was therefore prepared. It was made 3 times as strong as Van Slyke's standard so that the total volume of the solution could be kept small. The procedure used was: 1 cc. of serum was measured into a large test-tube (approximately 25 × 200 mm.), 1 cc. of the standard aqueous silver nitrate solution added, and the tube shaken; then 3 cc. of concentrated nitric acid were introduced, and, after being mixed, the contents were digested. Some digestions were carried out by heating the tubes in a boiling water bath for 2 hours and others by boiling the solutions gently over a micro burner for 15 to 20 minutes. After a few determinations were carried out and good results obtained, a series of comparisons was made with (1)

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the regular Van Slyke procedure with digestion for 2 hours and 16 hours, (2) aqueous silver nitrate and nitric acid with digestion in a water bath or over a free flame, (3) the Whitehorn method [1] (2).

Besides these micro methods, determinations with two macro methods were run in the hope that these might yield more accurate results and thereby constitute standard determinations with which to compare the results of the micro procedures. The methods were: (1) the Parr bomb method, which consists of burning dried organic material mixed with sodium peroxide in a Parr bomb and then determining the chloride gravimetrically. 10 cc. of serum were evaporated to dryness in a Parr bomb, the residue mixed with 10 gm. of sodium peroxide, and the determination carried out as described by Parr (3). (2) Alkaline ashing: 10 cc. of serum were evaporated to dryness in a platinum dish after the addition of from 0.5 to 1 gm. of sodium carbonate. The dry residue was carefully ashed by the Stolte (4) procedure. The white ash was dissolved in about 100 cc. of water, filtered, and the chloride precipitated by the regular procedure with nitric acid and silver nitrate. The precipitated silver chloride was filtered in a Gooch crucible, dried, and weighed. Table I gives the results of these series of analyses.[2] It may be seen that good agreement was obtained with all of the procedures except the Van Slyke which yielded results lower than the others.

When these results were called to Dr. Van Slyke's attention he carried out a series of analyses on human sera and failed to find such differences. Through his cooperation, Dr. Alma Hiller, his assistant, came to our laboratory and carried through a series of analyses with us. We then learned for the first time that Van Slyke's laboratory had used 50 cc. flasks instead of tubes on account of the convenience in titrating. A comparison was, therefore, made with both flasks and tubes with the Van Slyke and the aqueous silver nitrate procedures. The results are shown in Table II. It will be observed that the use of tubes with the Van Slyke

[1] 1 cc. of our standard aqueous silver nitrate solution was used instead of the silver standard recommended. No correction was subtracted from the thiocyanate used to titrate the excess of silver.

[2] The macro determinations reported in this paper were made in duplicate, the micro determinations in triplicate, except Experiments 4 and 5 on whole blood which were made in duplicate.

method yielded lower values than those obtained with flasks. However, the aqueous silver nitrate yielded still higher results. With the latter procedure no difference was noted between the results obtained with tubes or flasks. Thus it would appear that the silver nitrate and nitric acid should be added separately.

TABLE I.

Chlorides in Serum (Mg. of NaCl per 100 Cc.).

	Dog serum.				Human serum.		Horse serum.	Beef serum.
Sample No.	6	7	8	9	1	2	2	1
Parr bomb	692	669	678	753	632	624		
Alkaline ashing		669	683	751				
Van Slyke, 2 hrs.	660	632	660	672	598	572	590	591
" " overnight	670	660	669	715	607	597	618	
Aq. Ag, boiled 20 min.	691	661	680	750	624	622	619	595
" " water bath 2 hrs.	688	661	675	753	624	621	618	
Whitehorn	688	667	690	756	639	624		

TABLE II.

Comparison of Tubes and Flasks (Mg. of NaCl per 100 Cc.).

	Human Serum 3.		Dog Serum 10.		Dog Serum 11.		Horse Serum 2.		Dog Whole Blood 3.	
	Flasks.	Tubes.	Flasks.	Tubes.	Flasks.	Tubes.	Flasks.	Tubes.	Flasks.	Tubes.
Van Slyke, 2 hrs.	610 H	603 B	626 H	617 B	740 B 738 H	676 B 699 H	611 H	611 B		471* B
Aq. Ag, water bath 2 hrs.	621 "	623 "	623 "	623 "	754 B 753 H	751 B 748 H	615 "	613 "	486 B	484 "

B, run by Mr. Ball. H, run by Dr. Hiller.
* 16 hours.

A few experiments were carried out to study the effect of varying the time of digestion. The method of Osterberg and Schmidt[1] (5) was also used for comparison. Osterberg and Schmidt add dilute nitric acid and then silver nitrate and titrate in the presence of the precipitated protein and silver chloride. The results are given in Table III.

We obtained good agreement with all of the procedures except

the regular Van Slyke method. The end-point of the titration when the protein precipitate is present, as in the Osterberg and Schmidt method and in our undigested aqueous silver nitrate procedure, is quite poor. The turbid solution takes on a muddy appearance before the end-point is reached. Osterberg and Schmidt recommend a very fleeting end-point. A permanent end-point may be obtained by adding 1 or 2 more drops of thiocyanate solution. After digestion for 15 minutes with aqueous silver

TABLE III.

Chlorides in Serum (Mg. of NaCl per 100 Cc.).

Dog Serum.

Sample No.	8		10		11		12		13	
	Flasks.	Tubes.	Flasks.	Tubes.	Flasks.	Tubes.	Flasks.	Tubes.	Flasks.	Tubes.
Van Slyke 2 hrs.		660	626	617	738	676	666	638	634	627
Aq. Ag, water bath 2 hrs.		675		622	754	750		661		633
" " " " 30 min.		679		622		752				
" " " " 15 "		680		621			662	661	635	633
" " direct.							668	668	645 p	642 p
								663 p		
Osterberg and Schmidt, direct	690		631		744		663	650	638	641
							656 p	638 p	629 p	629 p
Osterberg and Schmidt, digested							665		629	
							655 p			
Whitehorn	690		632		759		658		635	

p = permanent color at end-point.

nitrate and nitric acid, the protein is sufficiently digested so that the end-point of the titration is just as sharp as that obtained after a 2 hour digestion, and the duplicates agree well. After making these comparisons we feel that the sharper end-point obtained after a short digestion is much more preferable than the end-point obtained with the Osterberg and Schmidt procedure.

In Table IV will be found a series of analyses of whole blood. On account of the dark color produced by the precipitated hematin the titration without digestion is obviously impossible. Good

determinations were obtained when titrations were made on solutions digested long enough to remove the brown color. When tubes were used the brown color had disappeared after 40 minutes digestion in a boiling water bath but 1 hour's heating in flasks on top of a boiling water bath was insufficient. The Van Slyke

TABLE IV.

Chlorides in Whole Blood (Mg. of NaCl per 100 Cc.).

Dog Whole Blood.

Sample No.	2	3	4		5	
			Flasks.	Tubes.	Flasks.	Tubes.
Alkaline ashing	532	489				
Van Slyke, overnight	515*	471*	501		482	
Aq. Ag, boiled 20 min.	527*	482*				
" " overnight	526*	478*	509		484	
" " water bath 40 min			498 a	508		484
			504 b			
" " " " 1 hr.					482	
" " hot plate 15 min.			507			
" " water bath 20 min.					474 a	475 a
Whitehorn	530	491	517		484	

a, solution brown. b, immersed in water bath.

* Determinations carried out in tubes only.

TABLE V.

Chlorides in Tissues (Mg. of NaCl per 100 Gm.).

Tissue.	Direct alkaline ashing.	Aq. Ag, boiled 30 min.	Van Slyke, 16 hrs.
Beef liver	134	130	125
" muscle	125	118	108

procedure which involves digesting overnight yielded results slightly lower than the others.

Table V gives the results of two series of analyses on tissues. The Van Slyke procedure yielded results lower than those obtained by alkaline ashing. With aqueous silver nitrate it is doubtful if maximum results can be obtained. We hope to carry out further studies along this line.

DISCUSSION.

It appears that there are some dog sera and many human sera with which the Van Slyke method will yield maximum results, especially if flasks are used instead of test-tubes. On account of the poor results frequently encountered and the dependence of the method on the type of container used for the determination, the modification in which aqueous silver nitrate and nitric acid are used in separate solutions seems preferable. In our aqueous silver nitrate procedure the silver nitrate is added before the concentrated nitric acid because, if the nitric acid is added first, chloride may volatilize from the concentrated acid solution. However, Osterberg and Schmidt add diluted acid before adding the silver nitrate. As the Osterberg and Schmidt method yields good results even though the end-point is poor, it would seem that the element of greatest importance is that the protein should not be precipitated at the same time as the silver chloride.

The procedure which appears to be most convenient and satisfactory is the modification of Van Slyke's method as follows: To 1 cc. of serum or whole blood are added with mixing 1 cc. of standard silver nitrate (0.15 N) and then 3 cc. of concentrated nitric acid. The mixture is digested until the protein has been dissolved (15 minutes are required for serum and 40 minutes for whole blood when tubes are immersed in a boiling water bath. Flasks containing whole blood may be digested in about 15 minutes on a hot plate just below the boiling point). After digestion is sufficiently complete 6 cc. of 5 per cent ferric alum are added and the solution is cooled to room temperature or lower: the colder the solution the sharper the end-point.[3] The solution is then titrated with 0.02 N ammonium thiocyanate with a micro burette and titration continued until 1 drop causes a color change which persists for about 1 minute at room temperature. The end-point is sharp. 0.02 to 0.04 cc. is subtracted from the titration to correct for the amount of thiocyanate required to give a suitable end-point in the presence of the silver chloride and thiocyanate, the nitric acid, and the water.

[3] We have tried to sharpen the end-point still more by using acetone and alcoholic thiocyanate as recommended by Smirk (Smirk, F. H., *Biochem. J.*, 1927, xxi, 31) without observing any definite improvement.

SUMMARY.

Van Slyke's method for chloride in blood or serum is modified by use of aqueous silver nitrate and nitric acid as separate reagents instead of the silver nitrate dissolved in nitric acid.

A critical study of various procedures was made.

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Note by Donald D. Van Slyke and Alma Hiller.

By the courtesy of Professor Wilson we have had the opportunity to test his improvement on the open Carius method, for blood chlorides, and we are in entire agreement with him that the reliability of the method is increased by adding the $AgNO_3$ in water solution, before the nitric acid. With horse sera we have never found that this change caused any difference in results. But in two human sera out of fourteen the results were 1 and 3 milli-equivalents per liter respectively (6 and 18 mg. of NaCl per 100 cc.) lower when our original procedure was followed, of adding the $AgNO_3$ dissolved in concentrated HNO_3 to the serum. We were unable to identify the serum constituents responsible for the abnormal results in the two cases. One was a lipemic serum, but the other was not. The method as improved by Wilson and Ball appears to give entirely correct values for sera of all types.

The writers take advantage of this opportunity to call attention to an earlier paper on the subject that was overlooked when the former paper was published, in which the first successful application of the open Carius principle to blood was ascribed to Greenwald. Laudat (*J. pharm. et chim.*, 1917, xvi, 168) had several years before published a method in which the organic matter of blood was destroyed by boiling with $KMnO_4$ and HNO_3 in the presence of $AgNO_3$, and the Cl was determined by titration of the excess Ag with NH_4CNS.

THE HEMOLYTIC ACTION OF INORGANIC ACIDS.

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(Received for publication, June 8, 1928.)

In his monograph "The Erythrocyte and the Action of Simple Hemolysins," Ponder (1) states that very little satisfactory work has been done on the hemolytic action of acids and alkalies. Reference to Jacobs' review in Cowdry's "General Cytology" (2) and to Höber's and Bayliss' discussions of hemolysis and the permeability of the red blood cell (3, 4), as well as to the work on hemolysis by Stewart (5) and others, confirms the impression that the subject has received scant attention.

The present paper reports certain observations that have been obtained in a study of hemolysis by the following acids: hydrochloric, sulfuric, nitric, and phosphoric.

Two types of corpuscles have been employed in this study; namely, those of the dog and man. The procedure employed in determining the time required for complete hemolysis of a standard cell suspension has been based on the method of Ponder (6).

Preparation of Red Blood Cell Suspension.—The cell suspension is prepared as follows: Immediately upon the drawing of 5 cc. of blood, they are added to 100 cc. of citrated saline (prepared by adding 15 cc. of 1.5 per cent sodium citrate to 85 cc. of 0.85 per cent sodium chloride). The cell suspension is then transferred to 50 cc. graduated centrifuge tubes. The cells are separated by centrifuging at a moderate speed, after which the supernatant liquid is removed and the cells again suspended in saline, this being added to the 50 cc. mark in each of the tubes. The process is repeated three times so that in all, the cells receive four washings with saline. Finally the cells are suspended in saline, making a suspension 100 cc. of which contain the corpuscles from 5 cc. of the original blood. The actual number of corpuscles per unit volume may be

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determined by count. The cells tend to settle out even on short standing. Accordingly when the suspension is used it should be mixed at frequent intervals.

Preparation of Acid Solutions.—In this work the acid solutions were prepared in 0.85 per cent sodium chloride. The stock solution was 0.1 N in strength, less concentrated solutions being prepared, as required, by dilution with physiological salt solution. A second set of solutions was prepared of the same acid concentrations but isotonic with 0.85 per cent sodium chloride. In the study of the effect of different concentrations of a given acid, the corresponding solutions were measured into small test-tubes of uniform diameter, 1.6 cc. being the volume used in each case. Determinations were usually made in triplicate. The pH of these solutions was determined potentiometrically, with a quinhydrone electrode. A volume of 16 cc. was usually found convenient. Since in the actual determination 0.4 cc. of the cell suspension is added to 1.6 cc. of the acid, the actual concentration of the acid at the beginning of hemolysis is the concentration of the acid after dilution with saline in the proportion: 4 parts of acid to 1 part of saline. Accordingly after an initial reading of the voltage was taken, 4 cc. of saline were added to the 16 cc. of the acid and a second reading taken, this reading being used in calculating the pH at the beginning of hemolysis.

Determination of Time Required for Complete Hemolysis.—All measurements were made at 25°. A large beaker nearly filled with water is fitted with a cover having a large hole through which a high form beaker is suspended. The cover should have three smaller holes, one for the thermoregulator, the second for a thermometer, and the third for a stirrer. The inside beaker likewise contains water and is fitted with a cover having three holes in a row, through which the test-tubes may be inserted, and two smaller holes, one for a thermometer and the second for a stirrer. A sheet of finely printed material (4 to 6 point type) is pasted around a part of the larger beaker, the letters facing the observer. In back of this screen a constant source of illumination should be placed.

The time required for hemolysis is determined with the aid of a stop-watch. Before a determination is made, the tubes containing the acid are brought to a temperature of 25° in a water bath.

When a determination is made one of the tubes is removed from the bath, 0.4 cc. of the red blood cell suspension is added rapidly, the contents mixed, and without delay the tube is suspended through one of the holes in the cover of the inside beaker. The procedure need not take more than 2 to 3 seconds; the stop-watch is pressed as the red blood cell suspension is added. The moment of complete hemolysis can be decided by comparison with a fully hemolyzed control tube placed in the bath. The time taken for a

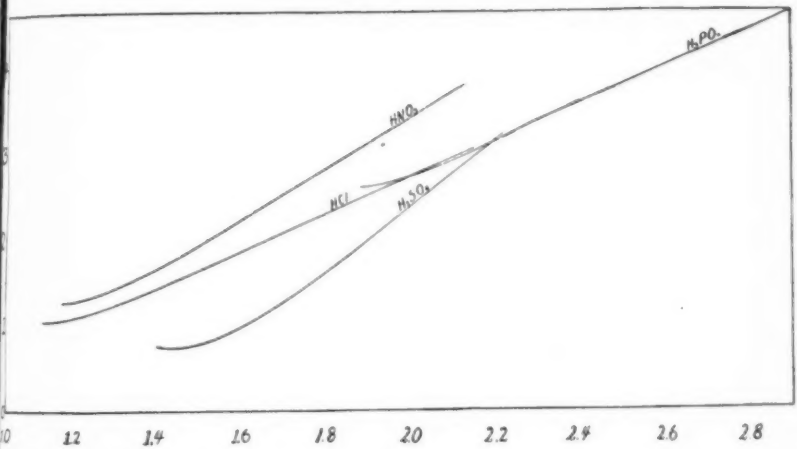

CHART I. Hemolytic action of inorganic acids. The curves show the relations between pH and rate of hemolysis of standard suspensions of dog erythrocytes.

particular dilution of acid to hemolyze 0.4 cc. of the blood suspension is then known. The method is very satisfactory, especially when hemolysis is relatively rapid.

Experimental Results.

In Chart I are represented the results obtained with hydrochloric, sulfuric, nitric, and phosphoric acids. These differ little in hemolytic action. The order of effectiveness in these experiments appeared to be: $H_3PO_4 > H_2SO_4 > HCl > HNO_3$.

Injury to the corpuscle rather than an osmotic effect seems to

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be the predominating factor in hemolysis by strong acids. In hemolysis by fatty acids, as will be shown later, an osmotic effect preceding hemolysis can be easily demonstrated. Jacobs (7) has shown that an osmotic effect is likewise responsible for the hemolytic action of the ammonium salts of the fatty acids.

TABLE I.

Effect of Tonicity of Acid Solutions on Hemolysis.

Concentration of acid.	0.1 N HCl.		0.05 N HCl.		0.1 N HNO_3.		0.05 N HNO_3.		0.1 N H_2SO_4.		0.05 N H_2SO_4.	
Tonicity	1.00	1.72*	1.00	1.36	1.00	1.70	1.00	1.35	1.00	1.40	1.00	1.20
Time, *min.*	1.02	0.87	1.50	1.38	1.25	1.20	1.83	1.74	1.00	0.75	0.83	0.92
Concentration of acid.	0.1 N H_3PO_4.		0.05 N H_3PO_4.		0.1 N propionic.		0.05 N propionic.		0.1 N butyric.		0.05 N butyric.	
Tonicity	1.00	1.15	1.00	1.075	1.00	1.38	1.00	1.19	1.00	1.38	1.00	1.19
Time, *min.*	2.60	2.45	2.95	2.90	1.25	2.70	3.67	4.33	1.05	2.10	3.67	4.78

* 1.72 × isotonic.

TABLE II.

Hemolysis of Standard Cell Suspensions. Comparison of Dog and Human Corpuscles.

Time required for hemolysis in minutes.

Acid	HCl				HNO_3			
pH	1.09	1.35	1.72	2.10	1.12	1.42	1.82	2.22
Dog	0.97	1.55	2.42	3.20	1.10	1.75	3.07	3.97
Man	0.43	0.77	1.92	3.00	3.50(?)	1.16	2.25	3.43
Acid	H_2SO_4				H_3PO_4			
pH	1.22	1.51	1.89	2.15	1.90	2.11	2.44	2.84
Dog	0.79	0.94	2.12	3.50	2.58	3.02	3.83	4.50
Man	3.50(?)	0.90	1.67	3.08	1.78	2.80	4.20	5.75

Corpuscles suspended in a dilute solution of a fatty acid in physiological salt solution are apparently subjected to the following changes: the fatty acid penetrates the corpuscle, and, depending on the amount of acid, is partly or completely neutralized by the buffer substances in the cell; water penetrates into the cell to equalize the osmotic pressure on the two sides of the membrane; the corpuscle swells and eventually bursts. If this is the mech-

anism, small differences in osmotic pressure on the two sides of the membrane should have an important effect on the rate of hemolysis. This is the case for the fatty acids, but in the case of hemolysis by inorganic acids, a moderate increase in the osmotic pressure of the outside fluid seems to have no effect in delaying the rate of hemolysis. In fact, in some instances, the cells suspended in hypertonic solutions hemolyzed somewhat more readily. This is brought out by the data in Table I.

TABLE III.

Hemolysis of Standard Cell Suspensions by Inorganic Acids. Comparison of Resistance of Corpuscles from Normal and Anemic Dog.

Time required for hemolysis in minutes.

Acid	HCl				HNO_3			
pH.	1.12	1.44	1.82	2.15	1.18	1.47	1.87	2.22
Normal dog	1.02	1.50	2.30	3.00	1.25	1.83	2.94	3.50
Anemic " *	1.00	1.50	2.30	3.23	1.25	1.78	2.87	3.67
Acid	H_2SO_4				H_3PO_4			
pH.	1.31	1.54	1.91	2.16	1.91	2.11	2.44	2.84
Normal dog	1.00	0.83	1.95	3.00	2.60	2.95	3.52	4.50
Anemic " *	1.05	0.88	2.08	3.22	2.33	2.88	3.70	4.67

* The red blood cell count of the anemic dog was 2.7 million per c. mm. as compared with a red blood cell count of 5.35 million for the normal animal. Accordingly, the cell suspension, in the case of the anemic dog, was prepared by use of twice the amount of blood taken normally. The number of corpuscles was therefore practically the same in the two suspensions.

Human corpuscles have a lower resistance to hydrochloric acid than dog corpuscles. For most concentrations within the effective range for rapid hemolysis this is also true for the other inorganic acids, as is indicated by the data in Table II.

It is interesting to observe that the resistance of corpuscles to inorganic acids does not change appreciably in experimental anemia produced by acetylphenylhydrazine, as is shown by the data in Table III. This result was quite unexpected in view of the known changes in the chemical composition of the red blood cell in this form of anemia.

TABLE IV.

Potentiometric Measurements during Hemolysis.

Series 11A. Dog corpuscles hemolyzed by hydrochloric acid.

0.1 N.		0.05 N.		0.02 N.	
Time.	E.M.F.	Time.	E.M.F.	Time.	E.M.F.
min.	*volts*	*min.*	*volts*	*min.*	*volts*
	0.3900		0.3735		0.3510
0.23	0.3845	0.25	0.3680	0.25	0.3440
0.50	0.3840	1.00	0.3660	1.00	0.3435
1.00	0.3840	1.20	0.3640	1.50	0.3400
2.00	0.3840	1.50	0.3640	2.00	0.3390
		2.00	0.3640	3.00	0.3390
Change.....	0.0060		0.0095		0.0120
Equilibrium reached in: 0.25–0.5 min.		1.2 min.		1.5–2 min.	
Observed time for hemolysis: 0.97 min.		1.55 "		2.42 "	

Series 12B. Human corpuscles hemolyzed by nitric acid.

0.05 N.		0.02 N.		0.01 N.	
Time.	E.M.F.	Time.	E.M.F.	Time.	E.M.F.
min.	*volts*	*min.*	*volts*	*min.*	*volts*
	0.3730		0.3525		0.3340
0.16	0.3676	0.16	0.3450	0.16	0.3250
0.33	0.3662	0.58	0.3445	0.50	0.3245
0.58	0.3655	1.00	0.3430	1.00	0.3240
1.00	0.3655	1.25	0.3425	2.00	0.3220
		1.40	0.3415	3.00	0.3180
		2.00	0.3415	4.00	0.3175
	0.0075		0.0115		0.0165
0.33–0.58 min.		1.25–1.4 min.		About 3 min.	
1.16 "		2.25 "		3.43 "	

Permeability of the Red Blood Cell to Inorganic Acids.

The red blood corpuscle resembles other types of cells, such as the sea urchin egg, in being seriously injured in moderate concentrations of inorganic acids. It is likely that the change produced by the acid renders the red blood cell membrane freely permeable to most of the cell constituents and is accompanied or followed by the disintegration of the framework of the cell. When a red blood cell suspension is added to an acid solution the buffer constituents of the corpuscle neutralize a portion of the acid. The neutralization may be followed potentiometrically. It occurs promptly when hemolyzed cells are added but when the cells are intact the process requires a longer time, depending on

TABLE V.

Hemolysis of Dog Corpuscles in Varying Concentrations of Hydrochloric Acid.

0.1 N.		0.001 N.		0.0001 N.		Control in saline.	
Time.	Red blood cell count per c.mm.	Time.	Red blood cell count per c.mm.	Time.	Red blood cell count per c.mm.	Time.	Red blood cell count per c.mm.
0	80,000	0	80,000	0	80,000	0	80,000
0.25 min.	68,000	10 min.	78,000	1 hr.	80,000	1 hr.	80,000
2 "	400	1½ hrs.	100	20 hrs.	60,000	4 hrs.	72,000
10 "	400						
3½ hrs.	350						

the concentration of the acid and the rate of hemolysis. The point of equilibrium is however the same in either case. When hemolysis occurs rapidly there is very close correspondence between the time required for hemolysis, as determined by the visual method, and the time it takes the reaction between the acid and cell buffers to reach equilibrium. It cannot be argued, however, that the acid reacts with the buffer after liberation from the cell and that neutralization follows hemolysis. The two processes overlap each other. In relatively slow hemolytic reactions neutralization is complete before hemolysis. The data in Table IV illustrate this point.

The red blood cell remains intact in a 0.0001 N solution of hydrochloric acid almost as long as in salt solution (Table V). Pre-

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sumably, even at this concentration the protein at the surface undergoes some change; the cell probably acquires a different electrical charge, but otherwise the toxicity effects are probably negligible. Even under these conditions acid diffuses into the cell and reacts with the cell buffers with considerable rapidity as shown by the data in Table VI. This is to be expected from what is known of the distribution of ions between the red blood corpuscle and its environment (Van Slyke, Wu, and McLean (8)).

Washed red blood corpuscles suspended in 0.001 N inorganic acid solution show practically no hemolysis during the first 10 minutes.

TABLE VI.

Neutralization of 0.0001 N HCl by Constituents of Red Blood Corpuscle.

Cell suspension.*		Control, hemolyzed cells.	
Time.	E.M.F.	Time.	E.M.F.
min.	*volts*	*min.*	*volts*
	0.1785		0.1785
0.16	0.1475	0.30	0.0910
0.42	0.1340	0.50	0.0820
0.75	0.1200	0.67	0.0755
1.00	0.1160	0.92	0.0720
2.00	0.1025	1.25	0.0675
3.00	0.0900	1.50	0.0660
4.00	0.0850	2.16	0.0630
6.00	0.0820	3.00	0.0630
13.00	0.0730	5.00	0.0630

* 4 cc. of a standard cell suspension were added to 16 cc. of acid. In the control, 4 cc. of hemolyzed corpuscles were added to 16 cc. of acid.

Yet, at the end of this interval the reaction between the acid and the cell buffers reaches completion. The curves in Chart II are based on the results obtained with the four inorganic acids studied in experiments in which both human and dog corpuscles were used. As in the preceding experiments the measurements were made at 25°, by means of a quinhydrone electrode. Quinhydrone does not seem to affect the rate of hemolysis. The acid (16 cc.) was measured into a beaker. The E.M.F. reading usually varied between 0.2700 and 0.2715 volts. The addition of 4 cc. of saline ordinarily produced a shift to about 0.2640 volts. This value was therefore taken to represent the initial hydrogen ion con-

centration of the solution. In the actual experiments 4 cc. of the standard cell suspension were added to the 16 cc. of acid, and readings were taken at short intervals. All the acids were ap-

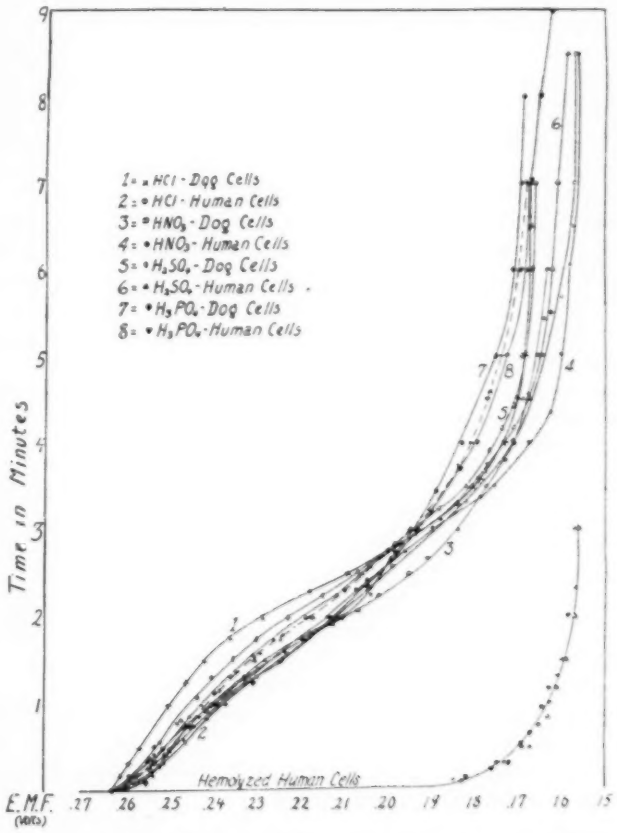
1 = HCl - Dog Cells
2 = HCl - Human Cells
3 = HNO3 - Dog Cells
4 = HNO3 - Human Cells
5 = H2SO4 - Dog Cells
6 = H2SO4 - Human Cells
7 = H3PO4 - Dog Cells
8 = H3PO4 - Human Cells
Time in Minutes
Hemolyzed Human Cells
E.M.F.
(Volts)
.27 .26 .25 .24 .23 .22 .21 .20 .19 .18 .17 .16 .15

CHART II. The curves represent the neutralization of various inorganic acids by the constituents of dog and human corpuscles. Curve 1 is based on the results obtained with hydrochloric acid, with a freshly prepared suspension of dog corpuscles The unnumbered broken line curve is based on results of a similar experiment with the same cell suspension after it had stood for 1 to 2 hours.

proximately 0.001 N in concentration, the phosphoric acid being 0.001 N with respect to the first hydrogen atom. The maximum shift in E.M.F. did not extend below 0.1560 volts in any of the experiments in this series. At 25°, this represents a pH of 5.01, at which point the dissociation of the second hydrogen atom of phosphoric acid is negligible.

It is to be seen from the curves in Chart II that the rates of penetration of the various acids into human and dog corpuscles did not differ greatly, although it is to be observed that in these experiments, hydrochloric and sulfuric acid penetrated the dog corpuscle somewhat more slowly than either phosphoric or nitric acid. The permeability of the washed corpuscle increases somewhat on standing. (Compare the broken and continuous lines for hydrochloric acid; the broken line is based on the results obtained with a cell suspension after it was allowed to stand for 1 to 2 hours.) The buffer content of washed human corpuscles was found to be greater than that of washed dog corpuscles. The same point of equilibrium was attained whether the reaction occurred between the acid and the constituents of the intact corpuscles or between the acid and hemolyzed corpuscles.

SUMMARY.

The order of effectiveness of the inorganic acids in producing hemolysis is: $H_3PO_4 > H_2SO_4 > HCl > HNO_3$.

In the concentrations necessary to produce rapid hemolysis (pH 1.0 to 2.8), injury to the cell membrane, rather than an osmotic effect, is the main factor responsible for the disintegration of the corpuscle by inorganic acids.

Human corpuscles have a somewhat lower resistance to inorganic acids than dog corpuscles.

The anemic and normal erythrocytes do not differ appreciably in their resistance to inorganic acids.

In hemolysis by acids, the reaction between the cell constituents and the acid is complete before hemolysis occurs. Inorganic acids react readily with the constituents of the intact corpuscle. This is partly due to the rapid diffusion, even from very dilute solutions, of inorganic acids, through the red blood cell membrane.

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LIPOID SOLUBILITY, PERMEABILITY AND HEMOLYTIC ACTION OF THE SATURATED FATTY ACIDS.*

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(Received for publication, June 25, 1928.)

Hemolysis by fatty acids is closely related to the ability of these substances to penetrate the red blood cell membrane. Jacobs (1) has studied hemolysis produced by ammonium salts of the fatty acids and found that these owe their hemolytic effect to the permeability of the corpuscle to ammonia and the fatty acids, both entering the cell with relative ease even in the undissociated form. The accumulation of ammonium salts of the fatty acids inside the corpuscle leads to the osmotic effects which ultimately result in the disintegration of the cell.

The penetration of fatty acids into living tissues has been the subject of many excellent investigations (Loeb, Harvey, Crozier, Taylor, and others), but with the exception of the work of Jacobs with the ammonium salts and the essentially qualitative study of Führer and Neubauer (2), the permeability of the erythrocyte to fatty acids and the hemolytic action of these substances have received little attention.

Loeb (3) studied the effect of different acids in the formation of artificial fertilization membranes in sea urchin eggs and found the order of effectiveness to be as follows: formic <acetic < propionic <butyric < caprylic < nonylic.

Harvey (4) investigated the penetration rates of different acids into the testis of the "prickly fish," which contains a purple water-soluble pigment sensitive to acids, and found that valeric acid penetrated much more rapidly than any of its lower homologues. Crozier (5) measured the penetration rates of various acids into

* A brief report of these observations was presented at the Ann Arbor meeting of the American Society of Biological Chemists, April, 1928.

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the mantle fold of the mollusc, *Chromodoris zebra* and noted the following order of effectiveness: valeric > butyric > propionic > acetic. The effectiveness of formic acid was intermediate between valeric and butyric acids, but in view of the fact that Crozier compared the action of solutions that were 0.01 N in strength, this result is not surprising since the dissociation of formic acid is considerably greater than of its homologues. Crozier has also studied sensory activation by acids (6), using for this work the common earthworm, *Allobophora*. The effectiveness of the various fatty acids was in the order: acetic <propionic < butyric < valeric <formic < caproic < caprylic. A summary of related investigations is to be found in Jacobs' review in Cowdry's "General Cytology" (7).

More recently Taylor (8) determined the threshold concentrations necessary to produce a sour taste and found that for the lower members of the saturated fatty acid series higher concentrations are needed than for the higher members. He found that for solutions of equal pH a high degree of sourness is associated with a high penetration velocity of the undissociated acid or of the anion. It is to be seen therefore that in all the phenomena that have been studied in which primary penetration of the acid is a factor, a given fatty acid is invariably more active than its lower homologues and less active than its higher homologues.

In the present investigation a comparison has been made of the hemolytic action of the members of the saturated series of fatty acids, from formic to capric, including isobutyric, isovaleric, and isocaproic acids. A sufficient number of concentrations in isotonic saline were employed in order to establish for each acid the relationship between hydrogen ion concentration and the rate of hemolysis. In this work, parallel studies were made with dog and human corpuscles. The methods employed have been described in an earlier paper (9).

Even a condensed tabulation of the results that have been accumulated would require a considerable amount of space. The data for the dog corpuscle are therefore represented by means of the curves in Chart I. The data for human corpuscles will be considered separately. These curves bring out for the various acids their relative hemolytic power, and for the individual acids the relation between dilution and the rate of hemolysis. The

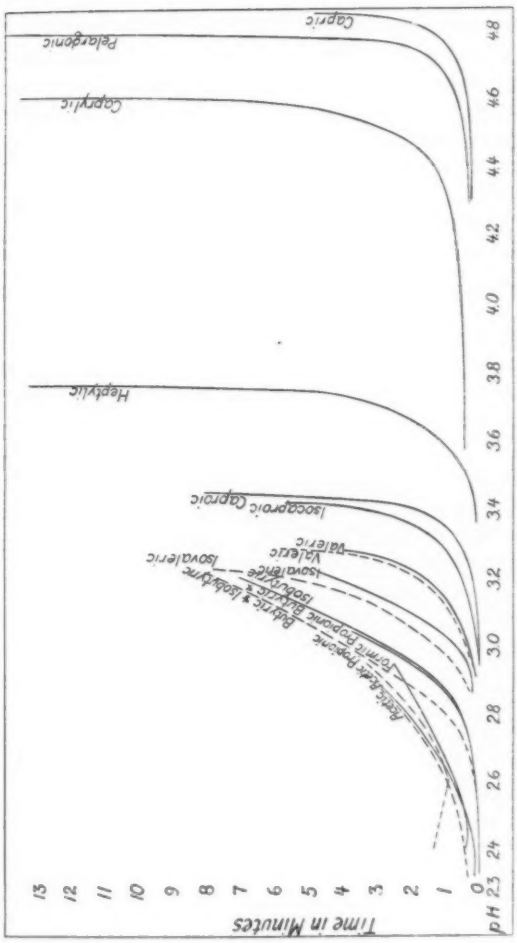

CHART I. Curves showing the relations between pH and the hemolytic action of the fatty acids. The determinations with caprie acid were made at 35°. All other determinations were made at 25°.

close parallelism of the results upon which these curves are based and the results of those who have studied the permeability of various tissues to acids makes it difficult to escape the conclusion that the permeability of the fatty acids is intimately related to their hemolytic effect. Formic acid resembles in its action more nearly the inorganic acids than the remaining members of its series. The character of its dilution curve sets it apart from the other fatty acids and the fact that moderate increases in the osmotic pressure of the outside fluid produce relatively little effect on the rate of hemolysis leads one to suspect that primary penetration of the acid is not the main factor involved in the hemolysis by formic acid, but that this acid resembles the inorganic acids in injuring the corpuscles in some other way (9).

Two curves are given for acetic acid. The broken line is based on the results obtained with solutions prepared in isotonic salt solution. The hemolytic action of acetic acid is low. It requires a normal solution to hemolyze a standard cell suspension in about 0.3 minute. Now, a normal solution of acetic acid in water is approximately 4 × isotonic, so that solutions of this acid, more concentrated than 0.25 N, even though prepared in water, are still hypertonic with respect to the cells. In view of the limited range of effectiveness of the acid, the concentrations above 0.25 N could not be excluded from the series, and the fact that these solutions were not isotonic is therefore to be borne in mind in interpreting the solid line for acetic acid, which is based on the results obtained with isotonic solutions of acetic acid of concentrations lower than 0.25 N and with solutions above this concentration that were unavoidably hypertonic. This applies also in the case of propionic acid, but for all the higher acids it was possible to adjust all the necessary concentrations to isotonicity. Two curves are given for propionic acid, the broken line being based on the data for the solutions prepared in saline and the solid line for the isotonic solutions. Two sets of curves are likewise given for butyric, isobutyric, valeric, and isovaleric acids in order to bring out the effect on the rate of hemolysis of small differences in the osmotic pressure of the outside fluid. The remaining curves are based on the results obtained with solutions prepared by dissolving the acid in 0.85 per cent sodium chloride. In the dilutions in which these acids were used, the addition of the acid to the physi-

ological salt solution was insufficient to produce any appreciable change in tonicity. A more detailed study of the relation of osmotic pressure to the rate of hemolysis is contemplated.

It will be observed that for a given pH, isovaleric acid is less effective than *n*-valeric acid and isocaproic acid is less effective than *n*-caproic acid in hemolyzing the corpuscles of the dog.

The higher fatty acids are very active hemolytic agents. These acids have a low solubility in water; the statements given in various handbooks that they are insoluble are, however, misleading. For example, a N/1500 solution of pelargonic acid is easily prepared. Pelargonic acid and its lower homologues are liquid at room temperature, and all the measurements with these acids were made at 25°. Capric acid, on the other hand, melts at 31°. The determinations with this acid were therefore made at 35°.

Under approximately uniform physiological conditions the properties of the red blood corpuscle of a given species are apparently such that experimental results of the type considered in this paper reproduce themselves from day to day with a uniformity that is very remarkable. Ponder (10) has observed this sort of consistency in his studies on saponin hemolysis. Corpuscles obtained from different normal individuals have approximately the same average resistance to saponin and the results which Ponder obtained with his own blood from time to time showed little variation. The present author has verified Ponder's assertions using his own blood, the data obtained being practically identical with those given by Ponder. Acids behave similarly in that over a long period of time there is little variation in the resistance of the corpuscles of a given animal towards a given acid. This does not mean, however, that the same mechanism is involved in the hemolysis by these two groups of substances; in fact the mechanisms are probably different.

The constancy in the behavior of the washed corpuscles to a given acid need not lead to the assumption that the permeability of the corpuscle is not subject to variation. The composition of the erythrocyte is far from being constant either physiologically or in a variety of pathological conditions. It is well known, for example, that the lipoids of the corpuscle are altered both qualitatively and quantitatively during fat absorption. Other changes

TABLE I.

*Data Showing Relative Resistance of Dog and Human Corpuscles to Varying Concentrations of Fatty Acids.**

Time for hemolysis in minutes.

Acid	Formic.				Acetic.				Propionic.			Butyric.			Isobutyric.		Valeric.		
Normality†	0.1	0.05	0.02	0.01	1.0	0.5	0.25	0.1	0.5	0.25	0.1	0.5	0.25	0.1	0.5	0.25	0.1	0.05	0.02
pH‡	2.39	2.52	2.73	2.93	2.32	2.47	2.63	2.85	2.58	2.70	2.92	2.49	2.67	2.86	2.52	2.68	2.89	3.06	3.27
Dog	0.36	0.62	1.25	2.30	0.38	0.63	1.17	2.75	0.95	1.25	2.70	<0.05	0.33	1.80	<0.05	0.50	0.07	0.85	5.75
Man	0.26	0.44	1.08	2.33	0.33	0.42	1.00	>4.00	1.22	3.42	>6.00	<0.05	5.75	>12.00	<0.05	7.00	0.11	2.08	>14

Acid	Isovaleric.			Caproic.				Isocaproic.				Heptylic.				Caprylic.		
Normality	0.1	0.05	0.02	0.1	0.05	0.02	0.01	0.1	0.05	0.02	0.01	0.02	0.01	0.005	0.002			
pH	2.86	3.03	3.22	2.92	3.05	3.24	3.42	2.89	3.03	3.22	3.42	3.36	3.43	3.61	3.78	4.33	4.46	4.53
Dog	0.23	1.83	7.75	<0.02	0.05	0.33	3.17	Instantly.	0.05	0.75	5.60	0.09	0.21	1.37	17	1.16	4.00	7.45
Man	0.94	>6.00	>13.0	0.02	0.06	2.00	17.0	0.02	0.07	3.15	>22.0	0.12	0.28	3.50	>35	2.58	12.5	>20

* The solutions used in these determinations were prepared by dissolving the acid in physiological salt solution.

† The normality values are close approximations and represent the concentrations of acid before the addition of the standard cell suspensions.

‡ The pH values represent the actual hydrogen ion concentrations at the beginning of hemolysis and were obtained in separate determinations by diluting the acid solutions with saline in the proportion of 1 cc. of saline to 4 cc. of acid. In determining the time required for complete hemolysis, 0.4 cc. of the standard corpuscle suspension is added to 1.6 cc. of the acid solution.

occur in experimental anemia. It is conceivable that changes such as these in the composition of the cell are likely to be associated with variations in the rate of penetration of various substances into the corpuscle.

Variations in permeability of acids would alter somewhat the position and shape of the dilution curves of the type given in Chart I, but despite such variations, the order of effectiveness of the different members of the series as represented by these curves will probably be found to hold under a large variety of conditions for all mammalian corpuscles.

A comparison of hemolysis of human and dog corpuscles by fatty acids has brought out certain distinctions. It will be recalled that human corpuscles are somewhat less resistant to inorganic acids than dog corpuscles. This is also true for formic and acetic acids. The resistance to propionic acid, on the other hand, is somewhat greater in the case of human cells, and for all the higher fatty acids the human corpuscles show a distinctly greater resistance to hemolysis than dog corpuscles. This is brought out by the data outlined in Table I.

The greater resistance of the human corpuscles appears to be related to their higher content of buffer substances. In measuring the shift in potential of the acid solutions during hemolysis, the washed human corpuscles invariably neutralized a greater amount of acid than dog corpuscles. In hemolysis with the lower fatty acids this effect is bound to be of relatively little importance because of the high concentration of acid needed to bring about hemolysis and of the relatively small proportion of this acid neutralized by the constituents of the cells. Yet, even in a 0.1 N solution of acetic acid the human corpuscles exhibit greater resistance than dog corpuscles. In the case of the higher fatty acids where lower concentrations suffice to bring about hemolysis, the difference in the neutralizing capacity of the constituents of the two types of cells may be sufficient to reduce appreciably the amount of available acid and lead to a reduction in the rate of hemolysis. It is to be seen from the data in Table I, as well as from the shape of the curves in Chart I, that a small decrease in the concentration of acid may result in a very pronounced retardation of hemolysis. For example, in the case of caproic acid, at pH 3.4, hemolysis of the standard cell suspension

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occurred in about 2 minutes, whereas when the initial pH was 3.45, complete hemolysis did not occur until after an interval of 8 minutes.

The data in Table II have been selected at random from the results obtained in the measurement of hemolysis by means of the potentiometer. The initial pH values of the acid solutions are given, as well as the values at the end of the reaction between the acid and cell constituents. While the differences in the pH shift between the human and dog corpuscles for the various acids is not great, nevertheless differences of like magnitude have a

TABLE II.

*Shift in pH Due to Neutralization of Various Acids by Constituents of Dog and Human Corpuscles.**

Acid	Acetic.	Propionic.	Butyric.	Valeric.	Isovaleric.	Caprylic.
Approximate normality	0.5	0.5	0.1	0.1	0.02	0.001
Dog.						
Initial pH	2.40	2.50	2.77	2.80	3.12	4.30
Final pH	2.58	2.73	3.12	3.11	3.62	5.02
Man.						
Initial pH	2.40	2.50	2.77	2.80	3.12	4.28
Final pH	2.63	2.80	3.18	3.17	3.73	5.20

* To 16 cc. of acid, 4 cc. of the standard red blood cell suspension were added.

considerable effect on the rate of hemolysis, especially for the higher fatty acids, as may be seen by projecting these values on certain portions of the curves given in Chart I.

Mechanism of Penetration of Fatty Acids into Red Blood Corpuscle.

In his study of cell permeability, Harvey (4) did not obtain clear cut evidence either to support or contradict Overton's well known lipoid solubility theory. Harvey states, however, that lipoid solubility, or capillary activity, for the two run more or less parallel, constitutes one of the factors upon which the penetration of acids depends.

Taylor's work on taste (8) has led him to conclude that sourness is not purely a function of the stoichiometric acid concentration, nor of the hydrogen ion concentration. In attempting to find an

explanation for his results he has assumed that the production of a given degree of sourness is due to the establishment within the cells of the taste bud of a definite hydrogen ion concentration and from his experimental results he has calculated the concentration gradients of equally sour acids. He recalculated similarly the taste data of Paul and Bohnen (see Paul (11)), and from the data of Crozier (5, 6) computed the concentration gradients of acids penetrating *Chromodoris* tissue, as well as the

TABLE III.

Concentration Gradients of Acids Penetrating the Red Blood Corpuscle and Producing Hemolysis of a Standard Cell Suspension in 1 Minute.

Acid.	pH	Total acid concentration.	H^+ ion concentration.	Concentration of undissociated acid outside. (a)	Relative concentration of undissociated acid inside. (b)	Ratio (a):(b).	Ratio (a):(b) when acetic acid = 1.
		$\times 10^{-3}M$	$\times 10^{-3}M$	$\times 10^{-3}M$			
Formic	2.67	23.5	2.14	21.4	1.00	21.4	
Acetic	2.64	286	2.3	284	11.5	24.7	1.00
Propionic	2.86	141	1.4	140	15.2	9.2	0.372
Isobutyric	2.87	122	1.35	121	14.3	8.46	0.342
Butyric	2.87	124	1.35	123	14.5	8.48	0.344
Isovaleric	3.03	51.9	0.93	51	12.6	4.05	0.164
Valeric	3.14	34.1	0.73	33.4	13.4	2.50	0.101
Isocaproic	3.28	19.2	0.53	18.7	14.3	1.31	0.053
Caproic	3.35	14.4	0.45	13.9	14.7	0.95	0.038
Heptylic	3.58	4.9	0.26	4.64	14.7	0.315	0.0128
Caprylic	4.33	0.2	0.047	0.153	14.9	0.0103	0.0004
Pelargonic	4.67	0.06	0.021	0.04	19.4	0.0021	0.000084

concentration gradients causing retraction in the earthworm. The resulting values, not only showed agreement with each other, but resembled Freundlich's data (12) on the relative concentrations of undissociated fatty acids (formic, acetic, propionic, and butyric) which are necessary in order that charcoal may adsorb the same quantity of acid in each case. Accordingly, Taylor suggests that the fatty acids are taken into tissues by an adsorption process.

It is probably unnecessary to postulate an adsorption mechanism, for the results of acid penetration into tissues can be more

fully correlated on the basis of solubility data which are outlined in this paper. In order to compare our results on hemolysis with the data that have been obtained by others in a variety of biological phenomena involving the penetration of acids into tissues, the relative concentration gradients of undissociated acids across the red blood cell membrane, causing hemolysis (*a*) in 1 minute, and (*b*) in 5 minutes, have been determined by the methods suggested in Taylor's paper. The results are outlined in Tables III and IV. The relative concentrations of undissociated acid

TABLE IV.

Concentration Gradients of Acids Penetrating the Red Blood Corpuscle and Producing Hemolysis of Standard Cell Suspension in 5 Minutes.

Acid.	pH	Total acid concentration.	H^+ ion concentration.	Concentration of undissociated acid outside. (a)	Relative concentration of undissociated acid inside. (b)	Ratio (a):(b).	Ratio (a):(b) when butyric acid = 1.
		$\times 10^{-3}M$	$\times 10^{-3}M$	$\times 10^{-3}M$			
Butyric	3.12	39.9	0.76	39.1	14.5	2.70	1.00
Isobutyric	3.12	39.3	0.76	38.5	14.3	2.70	1.00
Isovaleric	3.23	21.1	0.59	20.5	12.6	1.63	0.60
Valeric	3.30	16.2	0.50	15.7	13.4	1.17	0.43
Isocaproic	3.42	10.0	0.38	9.6	14.3	0.67	0.25
Caproic	3.44	9.3	0.36	8.9	14.7	0.60	0.22
Heptylic	3.75	2.3	0.175	2.1	14.7	0.143	0.053
Caprylic	4.58	0.073	0.026	0.047	14.9	0.0032	0.0012
Pelargonic	4.78	0.043	0.017	0.026	19.4	0.0013	0.0005

inside the cell are based on the dissociation constants of the fatty acids, as given in the "Landolt-Börnstein Physikalisch-chemische Tabellen." The present writer has been unable to find data for the dissociation constant of capric acid; the results for this acid are therefore omitted from the tables.

The ratios given in the last column of Tables III and IV may be taken to represent the relative gradients or driving forces which are necessary to cause the various fatty acids to penetrate into the corpuscles of the dog to a comparable degree. When allowance is made for the higher concentrations of acid necessary to

bring about hemolysis than are required to produce a sour taste or to cause sensory activation as in Crozier's experiments (6), the relations indicated by the data in Tables III and IV nevertheless bear a close resemblance to the relations that have been shown to exist in other biological phenomena which depend upon the primary penetration of the acids into tissues. That the primary penetration of the fatty acids into the erythrocyte depends on their solubility in the lipoids of the cell will now be considered.

It is generally known that the lipoid solubility of the members of the saturated series of fatty acids increases according to the size of the molecule. However, adequate data bearing on the distribution of the fatty acids between water and fatty substances seem to be lacking. In his paper on the permeability of cells to acids, Harvey (4) includes certain data obtained from Wasteneys on the partition coefficients between water and olive oil of several of the fatty acids in 0.01 N concentration.[1] In view of the possible relationship between the penetrability of the fatty acids into the corpuscle and lipoid solubility and because of the lack of solubility data, it seemed desirable to determine the partition coefficients of the fatty acids between water and olive oil. (The same results were obtained with aqueous solutions as with solutions of the fatty acids in saline.)

The solubility of the fatty acids was determined at 23°. 100 cc. portions of the various acids of known concentration (N, 0.1 N, 0.01 N, 0.001 N) were transferred to flasks. After the removal of 25 cc. for the preliminary titration with alkali, 5 cc. of olive oil (Squibb) were added to the remainder; the flasks were tightly stoppered and the contents shaken in a mechanical shaking device for 1½ hours. As a rule the distribution was found to reach equilibrium within 30 minutes. After the oil was allowed to separate, a second 25 cc. portion of the solution was titrated. The oil was then transferred to narrow test-tubes and set aside for 24 hours or longer to insure complete separation of the oily layer. Accurately measured portions of the oil were then dissolved in neutral alcohol and titrated with standard alkali. Suitable control analyses were made for the acid content of the olive oil. Excellent agreement was obtained in nearly all of the experiments

[1] Except for Harvey's citation, this work has not been published, according to a personal communication from Dr. H. Wasteneys.

TABLE V.

Solubility of Fatty Acids in Olive Oil.

Acid.	Acid lost from 75 cc. water and recovered in 5 cc. oil.	Acid in 1 cc. water at equilibrium. C_1	Acid in 1 cc. oil at equilibrium. C_2	Oil: water distribution ratio. $C_2:C_1$	Water: oil distribution ratio. $C_1:C_2$
1.0 N.					
	cc.	cc.	cc.		Formic acid = 1.
Formic	0.11	0.9985	0.022	0.022	1.00
Acetic	0.15	0.998	0.03	0.030	0.73
Propionic	1.05	0.986	0.21	0.213	0.104
Butyric	7.5	0.900	1.5	1.67	0.013
0.1 N.					
					Formic acid = 1.
Formic	0.025	0.9997	0.005	0.005	1.00
Acetic	0.15	0.998	0.03	0.03	0.165
Propionic	0.7	0.991	0.14	0.141	0.035
Isobutyric	3.1	0.959	0.62	0.645	0.0077
Butyric	3.1	0.959	0.62	0.645	0.0077
Isovaleric	11.0	0.853	2.2	2.58	0.0019
Valeric	17.2	0.77	3.45	4.48	0.0011
0.01 N.					
					Propionic acid = 1.
Propionic	0.75	0.99	0.15	0.1515	1.00
Isobutyric	2.15	0.972	0.43	0.443	0.342
Butyric	2.15	0.972	0.43	0.443	0.342
Isovaleric	8.5	0.887	1.7	1.92	0.079
Valeric	11.5	0.847	2.2	2.60	0.058
Isocaproic	26.0	0.653	5.2	7.96	0.019
Caproic	29.0	0.613	5.8	9.47	0.016
Heptylic	57.5	0.233	11.5	49.3	0.003
0.001 N.					
					Butyric acid = 1.
Propionic	0	1.00	0		
Isobutyric	1.5	0.98	0.3	0.306	1.00
Butyric	1.5	0.98	0.3	0.306	1.00
Isovaleric	7.5	0.90	1.5	1.67	0.184
Valeric	9.0	0.88	1.8	2.05	0.150
Isocaproic	16.5	0.78	3.3	4.23	0.072
Caproic	22.5	0.70	4.5	6.43	0.048
Heptylic	40.0	0.47	8.0	17.0	0.018
Caprylic	51.0	0.32	10.2	31.9	0.0095

between the amount of fatty acid lost from the aqueous layer and the amount of fatty acid recovered in the oil. The results, which are based on several series of determinations, are outlined in Table V.

Owing to the low solubility of pelargonic acid, concentrations lower than 0.001 N were employed. With such dilutions, the procedure of titrating the acid solutions before and after shaking with oil was subject to considerable error. The more accurate

TABLE VI.

Solubility of Fatty Acids in Olive Oil.

Acid.	pH shift.		Total acid concentration.		Acid lost from 75 cc. water.	Acid in 1 cc. water at equilibrium.	Acid in 1 cc. oil at equilibrium.	Oil:water distribution ratio.	Water:oil distribution ratio.
	Initial pH.	pH at equilibrium.	Initial $\times 10^{-4}$ M.	At equilibrium $\times 10^{-4}$ M.		C_1	C_2	$C_2:C_1$	$C_1:C_2$
					Cc. 0.000407 N.				
Isocaproic.....	4.15	4.23	0.407	0.291	21.5	0.713	4.30	6.04	0.166
					Cc. 0.000375 N.				
Heptylic.....	4.17	4.35	0.375	0.184	38.2	0.49	7.64	15.6	0.064
					Cc. 0.000443 N.				
Caprylic.....	4.14	4.64	0.443	0.060	64.85	0.135	12.97	96.1	0.014
					Cc. 0.000363 N.				
Pelargonic.....	4.24	5.02	0.363	0.00934	73.125	0.025	14.625	585	0.0017

procedure was therefore adopted of measuring the change in hydrogen ion concentration. Determinations were made with solutions of isocaproic, heptylic, caprylic, and pelargonic acids of approximately the same concentration. The results are given in Table VI.

An examination of the distribution ratios given in Tables V and VI and comparison of these data with the relative concentration gradients given in Tables III and IV, as well as with the other

data contained in this paper, lead to the conviction that lipoid solubility is an important factor in determining the rate of penetration of the fatty acids into the erythrocyte and the order of effectiveness of the various members of the series in producing hemolysis.

Since the data outlined in this paper are not difficult to correlate, a detailed discussion seems unnecessary, but for the purpose of illustrating how well the results agree, the following example will be considered. It is to be noted that the distribution ratios for butyric and isobutyric acid are identical for the concentrations included in Table V. The hemolysis curves (Chart I) are likewise identical, and accordingly the relative concentration gradients for these acids are the same (Table III and IV). On the other hand, the position occupied by isovaleric acid in the order of effectiveness as a hemolytic agent is intermediate between butyric and *n*-valeric, and isocaproic acid occupies a position intermediate between valeric and *n*-caproic acids. These are precisely the positions occupied by the oil : water (and water : oil) distribution ratios of isovaleric and isocaproic acids.

Incidentally, it is to be observed that the oil : water distribution ratios $\frac{C_2}{C_1}$ for the members of the series above propionic acid increase with increasing concentration of the acids. This indicates that the degree of molecular association of the fatty acids is greater in olive oil than in water. It is likely that this is also true for formic, acetic, and propionic acids, but in view of the low solubility of these in oil, the methods employed in this work were inadequate to bring this out with certainty.

SUMMARY.

The order of effectiveness of the fatty acids in penetrating the red blood corpuscle and producing hemolysis is: acetic < propionic < butyric = isobutyric < isovaleric < valeric < isocaproic < caproic < heptylic < caprylic < pelargonic < capric.

The relations between pH and hemolytic action have been determined for each of these fatty acids and the results represented by means of curves.

Increasing the osmotic concentration of the outside fluid retards

hemolysis by fatty acids. This shows that osmotic effects are involved in the process.

Formic acid resembles the inorganic acids in its hemolytic action.

Human corpuscles are more resistant to hemolysis by fatty acids than dog corpuscles. This is apparently due to the greater content of buffer substances in washed human corpuscles than in washed dog corpuscles and the neutralization of a greater amount of acid.

Determinations have been made of the relative concentration gradients of the acids, penetrating the corpuscle and producing hemolysis. The relations which are shown to exist in hemolysis resemble closely the relations that have been observed by other workers in a variety of phenomena in plant and animal tissues involving the primary penetration of the acids.

Determinations have been made of the distribution of the fatty acids between water and olive oil. A close parallelism exists between lipoid solubility and the effectiveness of the fatty acids in producing hemolysis.

The writer wishes to express his appreciation to Dr. B. M. Hendrix of this laboratory for a number of valuable suggestions.

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RICKETS IN RATS.

VII. METABOLISM OF CALCIUM AND PHOSPHORUS OF RATS FED UPON NON-RICKETOGENIC DIETS.*

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To evaluate the metabolism of calcium and phosphorus, in our previous experiments animals on various deficient diets have been compared to "normals." The standards adopted for calcium and phosphorus metabolism were those of Sherman for analyses of rats, of the Wistar Institute for bone analyses, and of the Lister Institute for metabolism studies. As their diets differed more or less widely from those we used, it seemed advisable to modify our diets toward normal by the addition of salts and vitamins, only one alteration being made at a time. In this way, we have been able to estimate, stepwise, the effect of the deficiency of the diet.

What constitutes normality is more or less a philosophical question. One is reminded of the ophthalmologist who said that in long years of experience he had seen but one normal eye. The usual meaning of the term is the median or mode of those individuals showing no known abnormalities. Chittenden and Mendel (1) emphasize that the average depends upon what is considered a proper diet. Experience with the albino rat has shown that our concept of the normal is changing.

Experiments of the Wistar Institute over a long period have resulted in gradual improvement of the average growth. Weight curves over a period of years have shown increasingly good animals (Osborne and Mendel (2)). Whether these animals represent

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accelerated early growth or actual increased size at maturity has not been determined. So far as known, neither of the laboratories mentioned has made special effort to select those animals for breeding which showed the best growth. Any conscious selection was to weed out the unfit. With regard to the nutrition of the animals, diets of the previous generation and especially of the mothers during pregnancy and lactation affect markedly the growth of the young. Even when these factors are kept constant, longer experience in handling animals gives progressively better results. Other standards—relative length and proportion of the body, the growth of special parts or organs—give results that differ from those of weight alone (3). In this study normality was judged by gross weight increases, and by histological appearance and ash content of the bones.

Our effort has been not to obtain the best possible growth, but so to adjust conditions that constant and reproducible experimental material was secured. We have tried to make animals ricketic under exactly the same conditions so that the curative effects of various procedures could be studied upon a standard ricketic animal. The quality or amount of protein, fat, or carbohydrate, has not been changed. Attention has been directed to slight alterations in the mineral composition of the diet and to the effect of vitamins. The diet, of course, should be considered as a whole: change in one constituent affects the relations of all the others. A diet which may be satisfactory with a given calcium content may be unsatisfactory if the protein, fat, vitamins, or any of the mineral elements is changed.

Plan of Experiment.—Rats reared in our laboratory were kept with their mothers until 21 days old. Mothers and young had access to the Sherman Diet B, which consists of two-thirds whole wheat and one-third whole milk plus 1.33 per cent of NaCl. Then the young were taken from the mother and continued on the same diet until 28 days old. They were then placed in metabolism cages of the Hopkins type and fed the diet to be tested. The animals were studied for the subsequent 35 days. In addition to general behavior and growth, the following were considered: blood serum analyses for calcium and phosphorus, histologic examination of the bones, analysis of the ash content of the fat-free bones, and metabolism of calcium and phosphorus.

Care of the animals, composition of the diets, and the analyses, were as previously described (4). Three to six animals in separate metabolism cages were used for each experiment, with approximately an equal number of males and females. The diet used to produce rickets was that of Steenbock and Black, Ration 2965 (5), which consists of 76 per cent yellow corn, 20 per cent gluten flour, 3 per cent $CaCO_3$, and 1 per cent NaCl. To 100 gm. of this mixture were added 10 gm. of lard. The calcium by analysis was 1.08 per cent and the phosphorus 0.254 per cent. The ratio of Ca:P = 4.1. 100 gm. of the ration are equivalent to 530 cc. of 0.1 N alkali. This was Diet A. It was altered by the addition of enough $NaH_2PO_4 \cdot H_2O$ to make the phosphorus

TABLE I.

Calcium and Phosphorus in Blood Serum of Rats Fed Modifications of Steenbock and Black's Ration 2965, for 35 Days.

Calcium.	Phosphorus.	Histological examination.	Diet.
mg. per cent	*mg. per cent*		
10.0	2.6	Marked rickets.	Steenbock's diet + 10 per cent lard (Diet A).
10.4	4.2	Normal.	Diet B (Ca: P = 2.0).
		"	Diet C (4.0 per cent butter).
11.4	8.0	"	Diet D (2 per cent cod liver oil).
12.9	6.9	"	Diet E (irradiated corn + 2 per cent cod liver oil).

0.54 per cent. The ratio of Ca:P was approximately 2.0. This ration per 100 gm. is equivalent to 392 cc. of 0.1 N alkali, and was called Diet B. Diet C was made from Diet B; 4 gm. of lard were replaced by 4 gm. of butter fat (tested as to its vitamin A content). Diet D was made from Diet B; 2 gm. of lard were replaced by 2 gm. of cod liver oil (tested for vitamins A and D). Diet E was made from Diet D; the corn was irradiated for 20 minutes at a distance of 30 inches with a Hanovia Alpine Lamp known to be active. The cod liver oil was fed separately.

Results.

Blood Serum Values.—Values of the blood serum for calcium and phosphorus, when the animals had been fed on the experi-

mental diets for 35 days, are shown in Table I. Diet A resulted in the low phosphorus characteristically associated with this rickets-producing diet. With the increase in phosphate content of the diet (Diet B), the phosphorus rose to a low normal value. Further addition of vitamins (Diets D and E) resulted in a further increase of phosphorus and also of calcium.

Histological Examination of Bones.—Changes in the salt composition of the diet were sufficient to produce a bone which on section was practically normal (Table I). Additional improvements in the diet were without further visible effect.

Bone Analyses.—Values of the bone analyses by the technique of Chick and Roscoe (6) are shown in Table II. The effect of the various diets is clear. The additions of an amount of phos-

TABLE II.

Analyses of Femora of Rats.

Six femora.

Diet.	Ca:P.	Age.	Weight.				Ash, per cent of fat-free.
			Wet.	Dry.	Fat-free.	Ash.	
		days	*gm.*	*gm.*	*gm.*	*gm.*	
A	6.0	61	1.56	0.671	0.540	0.168	31.6
B	2.25	65	1.64	1.035	0.881	0.530	60.1
C	1.9	65	1.45	0.876	0.760	0.358	47.0
D	2.7	65	2.37	1.321	1.202	0.725	60.4
E	2.40	65	2.65	1.463	1.334	0.766	57.6

phorus sufficient to make the ratio of Ca:P = 2.0, or optimal, resulted in bones normal as to percentage composition of the ash. When butter fat was fed for 4 weeks, the bone obtained was slightly less good. Cod liver oil and cod liver oil plus irradiated diet did not increase the per cent of ash in the bone above normal, but gave a larger bone. With the exception of the butter fat diet, increase in the size of the bones is progressive from Diet A to Diet E. The maximal effect is obtained not, as might be expected, with cod liver oil, but with cod liver oil supplemented with irradiated corn.

Metabolism.

Weights of Animals.—Although the rats had free access to the food at all times the different groups grew at different rates.

The individuals in each group were so nearly alike that only the averages are shown in Table III. On the rickets-producing diet they grew poorly, actually losing weight for the last 2 weeks. The rate of growth improved progressively from the slowest, when the rats were given Diet A, to the fastest when they received

TABLE III.

Gain in Weight, Food Intakes, and Feces of Rats Fed Ricketogenic and Non-Ricketogenic Diets.

Diet.	Initial weight.	Final weight.	Period No.					Average.
			1	2	3	4	5	
Gain per wk. per rat.								
	gm.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
A	48	63	7.0	7.0	7.0	−3.0	−2.0	3.0
B	52	72	2.3	4.0	6.7	2.8	5.1	4.2
C	43	63	*	6.7	3.7	5.1	1.1	4.1
D	60	105	8.8	12.5	11.1	6.1	6.0	8.9
E	56	128	11.7	16.7	15.0	14.0	14.1	14.3
Net food per wk.								
A								30.8
B			32.9	35.0	36.4	33.6	33.6	34.3
C			*	35.0	32.2	30.8	25.9	30.8
D			53.9	56.0	53.9	60.9	41.3	53.2
E			52.5	66.5	64.4	74.2	71.4	65.8
Feces, dry weight per wk.								
A								3.6
B			3.58	3.59	3.57	3.43	3.96	3.62
C			*	2.71	3.69	3.54	3.43	3.34
D			5.24	5.65	6.26	5.94	5.02	5.62
E			5.32	7.66	6.16	7.70	7.92	6.95

* Group C was fed on Diet B for the 1st week.

Diet E. The maximal effect was obtained not when cod liver oil was added to a diet containing a good salt mixture, but when this diet was further improved by irradiation. The combined addition of light and cod liver oil to the diet resulted in normal growth. With Diet A the animals gained only for the first 3 weeks. With Diet B there was moderate growth throughout the

TABLE IV.

Metabolism of Calcium and Phosphorus.

Figures are in terms of one rat per week.

Diet.	Period.	Calcium.							Phosphorus.							
		Intake.	Urine.	Stool.	Spill.	Total output.	Balance.	Per cent.	Intake.	Urine.	Stool.	Spill.	Total output.	Balance.	Per cent.	Ca:P.
	wks.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	
B	I	365	11	178	+6	205	+160	44	163	11	87		98	+65	40	2.47
	II	392	13	218	+9	240	+152	39	175	7	107	+2	116	+59	34	2.57
	III	393	19	218	+6	243	+150	39	195	11	110		121	+74	38	2.03
	IV	365	11.6	221	+11	248	+117	32	181	7	117	+4	128	+53	29	2.20
	V	362	18	267	+5	290	+72	20	179	7	143	+2	152	+27	15	2.67
Average...		375	14.7	251		245	130	35	179	8	113		123	56	31	2.38
C	II	394	15	159	+10	184	+210	53	205	11	73	−3	81	+124	60	1.69
	III	362	20	213	+10	243	+119	33	188	17	103	−2	118	+70	37	1.70
	IV	344	18	215	+6	239	+105	31	179	13	109	−1	121	+58	32	1.80
	V	291	21	188	+2	211	+80	27	152	14	95		109	+43	28	1.85
Average...		348	18	194		219	129	37	181	14	95		107	74	41	1.76
D	I	558	49	256	+75	380	+178	32	209	6	88	+2	96	+113	55	1.58
	II	585	58	316	+47	421	+164	28	218	7	126	+5	138	+80	38	2.05
	III	564	76	346	+36	458	+106	19	210	4	154	+1	159	+51	24	2.08
	IV	631	55	317	+16	388	+245	39	236	3	131	+2	136	+100	41	2.45
	V	433	48	291	+16	355	+88	20	162	3	115	+1	119	+43	25	2.05
Average...		554	57	363		400	156	28	207	5	123		130	77	27	2.04
E	I	560	64	275	+30	369	+191	34	235	5	100	−2	103	+132	56	1.45
	II	710	93	391	+84	568	+142	20	299	4	153	+5	162	+137	46	1.03
	III	693	61	333	+42	436	+257	37	291	5	137	−1	131	+150	52	1.71
	IV	850	104	434	+65	603	+247	29	358	7	170	−12	165	+193	55	1.28
	V	749	103	457	+65	625	+124	17	282	5	169	+3	177	+105	37	1.18
Average...		712	85	378		520	192	27	293	5	146		149	144	49	1.33

5 weeks. When butter was added (Diet C) moderate growth took place for only 1 week. At the end of the period the animals were definitely failing. Cod liver oil (Diet D) gave its best results for the first 3 weeks. Diet E containing cod liver oil plus irradiated corn, not only gave the best growth, but the rate of growth was maintained throughout the experimental period.

Food Intakes.—Consumption of the diets containing added phosphate and butter was similar to that previously reported for the rickets-producing diet, 30 to 40 gm. per week. When cod liver oil was added more food was consumed; when irradiation was combined with cod liver oil still more food was eaten. Animals on the basal diet ate approximately one-half the amount

TABLE V.

Distribution of Ingested Calcium and Phosphorus.

Diet.	Calcium.			Phosphorus.		
	Urine.	Feces.	Retention.	Urine.	Feces.	Retention.
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
A	17	57	25	4	69	27
B	4	67	35	4	63	31
C	5	52	37	8	51	41
D	10	55	28	3	60	37
E	12	53	22	2	49	49

consumed by those fed upon the irradiated diet plus cod liver oil. The data are given in Table III.

Weight of Feces.—The dried feces increased progressively in weight from Diet A to Diet E except for the low values on the diet containing butter (Table III). These values correlate exactly with the gains in weights of the animals and the food consumption. They demonstrate, further, approximately the same degree of utilization; for in all cases, the dry feces equal 10.5 to 11.5 per cent of the weight of the food eaten.

Metabolism of Calcium and Phosphorus. Paths of Excretion.—The data for the intakes and outputs of calcium and phosphorus are given in Tables IV and V. Compared with the rickets-producing diet (7), Diet B, containing added phosphate, caused less calcium to be excreted in the urine. Calcium excretion in the feces remained approximately the same. The phosphorus ex-

cretion, on twice the intake, was approximately doubled in both urine and feces. When the diet was further improved (Diets C, D, and E) both urinary and fecal calcium increased. The phosphorus in the feces was increased slightly and that in the urine was constant or diminished. Because the intakes of both elements were progressively increased with the last three diets, such a comparison may be misleading. The relations are perhaps more clearly brought out when the excretions are compared with the intakes. 4 to 12 per cent of the calcium was in the urine, 52 to 67 per cent in the feces. 3 to 14 per cent of the phosphorus was in the urine and 49 to 67 per cent was in the feces. Table V reveals that the relative percentage of calcium and of phosphorus excreted by the urine and feces is roughly the same for all the diets.

Balances of Calcium and Phosphorus.—The positive balances increased progressively from Diet A to Diet E (Table IV). Each of the diets with added phosphorus resulted in a retention not only of more phosphorus but also more calcium. As the diets were improved a smaller percentage of calcium was retained and greater percentage of phosphorus, so that the ratios of retention fell progressively from 4.0 on the rickets-producing diet to 1.33 on the diet containing both irradiated corn and cod liver oil. Difference in the actual amounts of calcium and phosphorus retained depends primarily upon the food intakes.

DISCUSSION.

Boas (8) uses normal control diets with the ratio Ca:P = 1.5 to 1.0. Medes (9) considers a normal Ca:P ratio 1.4 or 1.5. McCollum (10) has normal diets with the Ca:P varying from 1.0 to 2.0, based upon analyses of the rat. Osborne and Mendel (11) give the ratio of 1.7 based upon the analyses of milk. In their later work (12) they have used 2.0, as a result of histological findings. Variations in the Ca:P ratio which these various authors consider normal imply that for the normal rat this factor is of minor importance, and only in absence of vitamin D, are small differences in the mineral constituents important. All of these ratios of Ca:P vary between 1.0 and 2.0. At the time when our experiments were performed the ratio of 2.0 was the best empirical value available.

That the curative agent in rickets was supplied completely either by the vitamin in cod liver oil or by the property conferred by irradiation, has been generally assumed. If either was supplied the other was unnecessary. Huston and Lightbody (13) have, however, shown that the growth of rats fed upon diets which contained adequate amounts of cod liver oil was improved either by hydroquinone or by irradiation. Our work confirms theirs and extends it. Both studies show that light and cod liver oil added to the diet result in double the rate of growth produced by the addition of either alone. Accordingly, the additional factor supplied by irradiation is a limiting factor for growth.

The metabolism studies show that retention of calcium and phosphorus are proportional to food intakes. The comparison of our data for retention with that of other investigators is difficult, because data for the age, gain, or food intakes or vitamin content are lacking (Telfer (14), McClendon (15), Medes (9), Hess (16)), or because the salt content of the diet was purposely made abnormal (Haag and Palmer (17)). Boas (8) has reported two sets of metabolism experiments in which all the data are included. The calcium and phosphorus retention in her animals supplied with cod liver oil closely approximates ours. The average ratios of retention of Ca:P were 1.72, 1.73, and 1.35, when the ratio of Ca:P of the diet was 1.5, and 1.56 when the ratio of the diet was 1.0. The diminution of the ratios of Ca:P in retention in our group that received both irradiated food and cod liver oil (Diet E) probably indicates that with good growth more phosphorus is utilized for growth of the tissues. In confirmation of our previous work and in agreement with Orr, Holt, Wilkins, and Boone (18), the ratio of Ca:P in retention is proportional to the ratio of the same elements in the diet.

SUMMARY AND CONCLUSIONS.

The diet has been progressively improved from a rickets-producing diet toward normal by alteration in the salt composition and by the addition of vitamins. A corresponding improvement occurs in the growth of animals, in the character of the bones, and in the retention of calcium and phosphorus. This improvement is brought about primarily by the increase in food consump-

tion. The best results ensue when the diet is supplemented with cod liver oil and by irradiation.

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RICKETS IN RATS.

IX. pH OF THE FECES.*

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Zucker and Matzner (1) have shown that the feces of ricketic rats are alkaline, pH 7.4, and that during the cure by cod liver oil the reaction becomes acid, pH 6.2. This has been corroborated by Jephcott and Bacharach (2) and also recently by Redman, Willimott, and Wokes (3). The former have suggested that this alteration in pH be used as a test for the cure of rickets. Variable findings have been reported by Redman (4) for children. The question then arises: Would other diets or other curative agents give different results, or is this a general method for testing the cure of rickets in rats?

Procedure.—Rickets was produced by Zucker's diet, or by the diet of Steenbock and Black, Ration 2965, plus 10 per cent lard. The procedure has been previously described (5). The ricketogenic diet was fed to rats at the age of 28 days. They continued on this diet for 21 days. Animals fed on Zucker's diet were then given cod liver oil. Those fed on Steenbock's diet were divided into groups of four or five animals. Cure was effected by the addition of cod liver oil, irradiation of the corn, irradiation and cod liver oil, or by the addition of phosphate sufficient to make the phosphorus equal in weight to, or double the weight of the calcium. The resulting diets, per 100 gm., were equivalent to 530 cc. of 0.1 N alkali, to neutrality, or to 315 cc. of 0.1 N acid.

These animals were studied for their responses to galvanic currents (6) and when tested, feces were produced and collected

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XUM

in clean test-tubes. Feces from the cages were never used. Thus the material was known to be fresh and uncontaminated. The pH was determined within an hour by the electrometric method. The chain consisted of a saturated calomel electrode, saturated KCl, agar bridge, test solution, and a gold-plated electrode with quinhydrone. The system checked daily against known solutions within 0.02 pH. Duplicates in close agreement were always obtained. As a further check the solutions were compared by the colorimetric method. This check was made independently by the second investigator.

Results.

The cure of rickets in rats by cod liver oil, irradiated food, and phosphates has been demonstrated by blood serum determinations for calcium and phosphorus, histological preparations of the bones, the ash content of the fat-free bones, and the metabolism of calcium and phosphorus (7). In this study it was further verified by x-ray pictures. Except for those animals which received high phosphate additions to the food and showed only partial recovery, all showed healed rickets after 2 weeks upon the curative measure.

The pH of the feces is tabulated in Table I. The data represent the average values of pH for all observations on a given day. The acidity determinations were so close that the pH and not C_H values were averaged. The feces of rats fed upon Zucker's diet, which had previously been alkaline, became acid on the 5th to 9th day after administration of cod liver oil. This confirms the work of previous investigators (1–3) with regard to the time required for, and the extent of, the change.

The feces of the animals which received Steenbock and Black's rickets-producing diet were alkaline. In this case also, in 5 to 9 days after the administration of cod liver oil, they became less alkaline and were practically neutral. The feces of the group which received irradiated food did not show the change, but remained alkaline. With a diet containing both irradiated corn and cod liver oil, the feces were practically neutral. The rats which were cured by the addition of acid phosphates produced acid feces beginning with the 2nd day. Those which ate the neutral diet had neutral or alkaline feces. Those which received alkaline diets produced alkaline feces.

TABLE I.

Fecal pH of Rats Cured of Rickets by Various Procedures.

Diet.	Ca:P in diet.	Potential acidity of diet.	Days on diet.	Average pH.
		cc. 0.1N per 100 gm.		
Zucker.	4.73	109 alkali.	14	8.1
			21	7.6
Zucker + cod liver oil.	4.73	109 alkali.	1	8.17
			2	7.76
			3	7.70
			5	6.53
			7	6.77
			8	6.41
Steenbock.	6.65	530 alkali.	14	7.41
			20	8.00
			22	7.43
Steenbock + cod liver oil.	6.65	530 alkali.	4	7.02
			6	6.93
			13	7.00
Steenbock irradiated.	6.65	530 alkali.	4	7.15
			6	7.45
			13	7.32
			17	7.68
Steenbock irradiated + cod liver oil.	6.65	530 alkali.	4	6.83
			5	7.18
			6	7.27
			9	7.28
			11	6.92
Steenbock neutral + phosphate.	0.90	35 alkali.	1	7.89
			2	7.08
			3	7.80
			4	7.48
Steenbock neutral + high phosphate.	0.50	15 alkali.	2	7.69
			3	7.60
			5	7.09
			7	7.08
			9	7.18

TABLE I—*Concluded.*

Diet.	Ca:P in diet.	Potential acidity of diet.	Days on diet.	Average pH.
		cc. 0.1N per 100 gm.		
Steenbock acid + phosphate.	0.90	350 acid.	1	7.55
			2	6.85
			3	6.88
			4	6.55
Steenbock acid + high phosphate.	0.50	350 acid.	1	7.76
			2	7.73
			3	7.31
			5	6.90
			7	6.85
Steenbock alkaline + phosphate.	0.90	860 alkali.	1	7.60
			2	7.21
			3	7.43
			5	7.04
			7	7.06
			9	7.48
			13	7.28

DISCUSSION.

The confirmation in several laboratories of Zucker's observation is interesting. However, as has recently been suggested by Coward (8), it seems hazardous to use the acidity of the feces as a test of antiricketic potency, even for cod liver oil preparations. The slight alteration in pH with Steenbock's diet as compared with the greater shift with Zucker's diet may be associated with their different potential alkalinities. The former, per 100 gm., is equivalent to 530 cc. of 0.1 N and the latter to 110 cc. of 0.1 N alkali.

Because the cure can be accomplished also in other ways than by ingestion of cod liver oil, the test is further limited. The published statements concerning the effect of irradiation are not necessarily in conflict with our own observations. Zucker (1) and also Jephcott and Bacharach (2) irradiated the rats as a preventive measure and found a constant acid pH. The ricketic rats of Jephcott and Bacharach that were irradiated showed "the

change in faecal pH illustrated" in their chart. The graph shows only a temporary drop below pH 7.0 for each of the two pairs of rats, and thereafter the feces are alkaline. Further, their findings cannot be compared directly to ours because they used Zucker's diet, which on irradiation, may result in a greater change in fecal pH than would a more alkaline diet.

The cure of rickets is not necessarily associated with an alteration of the pH of the feces from alkaline to acid. The laws which govern the reaction of the feces are not well understood. The calcium, phosphorus, and fat content must be important (9). Certainly, the effect of variations in the acid-base value of the diet on pH of the feces must be taken into account.

SUMMARY.

Alteration in the feces from alkaline to acid has been observed when rats fed on Zucker's diet are cured of rickets by cod liver oil. Although the change is of great interest in interpreting the mechanism of the action of cod liver oil, the cure due to other agents may not render the feces acid. This change does not occur when rats made ricketic on Steenbock's diet are cured by irradiation of the food or by the addition of alkaline phosphates.

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A COMPARISON OF THE BERGEIM AND STANDARD METHODS OF DETERMINING COEFFICIENTS OF UTILIZATION WITH SUGGESTED MODIFICATIONS.

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The customary proximate food analysis often fails to serve as an index of the feeding value of a ration. More satisfactory methods, such as the determination of the coefficient of utilization of any particular constituent of the feed, have long been recognized. The progress in the improvement of the method since its introduction by the German workers, and the frequent discussions of its application are so well known that a review of the literature at this time is not necessary. Armsby (1), who has made extensive application of these methods in this country, has outlined the most satisfactory steps of procedure. These methods are not used so extensively, however, as the information obtained would warrant, because of the tedious work involved as well as the many opportunities for error; namely, determining the accurate amount of food eaten, obtaining an equilibrium in the animal's body, collecting the total amount of feces and urine, and the preserving of the samples until the time of analysis. Bergeim (2) has recently suggested a method which eliminates many of the long, inaccurate steps of the old methods, and one, which if found to correlate well with the previous methods, might find extensive application. This method briefly consists in adding an insoluble iron compound to the ration and calculating the utilization coefficient on the basis of the ratio between the percentage of iron and the desired constituent in the feed, compared to that of the same two substances in the feces. For certain studies that we were attempting to make, this method would have proved far

superior if we had been sure that our results were comparable with the standard method, but an investigation of the literature failed to provide such information. In order to check these points we have attempted to make these determinations on a large number of animals, running the two methods in parallel, noting particularly whether the coefficient of utilization calculated by the two methods were comparable; second, whether the iron were all eliminated in the feces; third, whether a period of time were necessary after starting the feeding tests before the animal's body reached an equilibrium with the iron; and fourth, whether the utilization of the animal might be affected by the presence of the iron, an element, the salts of which are often administered for therapeutic purposes. Bergeim (2) bases his method on the commonly accepted view that ferric oxide is eliminated quantitatively in the feces and that it has no effect upon assimilation. On the other hand, Wada (3) states that possibly ferric oxide has the power to increase assimilation.

EXPERIMENTAL.

In order that the two methods could be accurately compared, it became necessary to choose test animals that could be kept under constant observation, and that their food and water should not be contaminated by contact with iron or its compounds. The rat was chosen as the most desirable animal. Metabolism cages containing no iron in their construction were next provided by cylindrical cages built of glass tubing set in concrete rings. The cage bottoms were similarly built of glass rods set in cement, the rims being enameled. Under this floor was placed an aluminum screen to catch the feces, while the urine was gathered in a porcelain dish below the aluminum screen. The greatest obstacle, that of a feeding jar from which feed could not be wasted, was overcome by securing a jar 6 inches long and $1\frac{1}{2}$ inches in diameter at the top. About the top was a 3 inch extension flange which offered a seat for the rats while eating and which caught any feed removed. The entire feeder was suspended by copper wire. In order to eat, the animal had to sit on this swinging seat and crawl head first into the jar. The position made it impossible to scratch out any food, and it was the only feeder ever used by the writer from which food was not wasted.

Distilled drinking water was provided through glass tubes from inverted bottles suspended on the outside of the cage. The animals used were healthy young males, females being omitted due to possibility of abnormal factors produced in pregnancy. In all cases the animals selected were kept on the ration being studied for several weeks prior to the time of the collecting of the first samples, so that a condition of equilibrium would be obtained in the animal's body and also to overcome any abnormalities that might be produced by a change in ration or quarters.

As previously stated, the work was so planned that the coefficient of utilization of the proteins, fats, fibers, and minerals could be determined by the Armsby method, the total feed and feces being analyzed, and at the same time the comparison of the coefficient of utilization based on the iron content of the feed and feces checked by the Bergeim method. In a parallel series the same determinations were being made, iron in the form of 0.5 per cent ferric oxide being added to the ration, the object being in this case to determine whether the presence of the iron had any effect on the utilization of the feed.

In the course of the study, hundreds of determinations have been made, a wide variety of rations as well as various iron salts being used. In this report, however, accounts will be made of rations similar in composition to that used for feeding purposes generally (Ration I); and of those similar in nature but having a protein supplement of more definite composition (Ration II).

The constituents of the rations and their analyses are as follows:

Ration I.

	parts
Yellow corn	60
Whole wheat	28
Tankage	5
Alfalfa leaves	5
NaCl	1
$CaCO_3$	1

Ration II.

	parts
Yellow corn	50
Whole wheat	45
Casein	10
Cod liver oil	2.5
NaCl	1
$CaCO_3$	1

Analysis of Ration I.

	per cent
Protein	14.93
Fat	2.99
Ash	7.19
Fiber	3.51
Water	9.96
Carbohydrates	61.42

Analysis of Ration II.

	per cent
Protein	16.8
Fat	4.44
Ash	2.85
Fiber	2.6
Water	8.6
Carbohydrates	64.71

XUM

It was soon found that the animals did not consume the same amounts of food daily and that the weight of feces did not parallel the food intake. This was due, no doubt, to changes in room temperatures and other factors. This has been noted of the food intake of an entire series of animals under experimental observation. For this reason, it was found advisable to take the food intake of a 4 day period and mix the feces and urine of the same period, although the samples were collected and preserved daily to prevent decomposition. The methods of analysis for protein, fat, moisture, ash, and fiber were those customarily used in feed control work and as adopted by the Association of Official Agricultural Chemists (4). In the case of iron determinations, however, many methods have been tried and various modifications introduced in an attempt to find some method that would be simple and at the same time uniformly accurate. The method recommended by Bergeim (2) was first used. Briefly stated it consists in taking a given weight of the sample and ashing at black heat, dampening with nitric acid and reashing, dissolving the residue in 5 cc. of 1:1 HCl and diluting to 10 cc., oxidizing with permanganate, adding 1 cc. of 2 per cent sodium thiocyanate, and comparing the color produced with a standard iron sample similarly and simultaneously prepared. This method has its limitations, and while its use has been checked on hundreds of determinations, it has been found usually to be low, especially in the analysis of the feces from the iron-consuming rats. It was first thought that the animals might be storing the iron or it might be excreted in the urine, but an analysis of the organs of the rats, and in fact of the whole animal, did not account for this loss. It was found that when the crucible in which feed was ashed was heated too rapidly the crucible often fused with some of the iron, while ashing at lower temperatures often left some organic matter. The method of Elvehjem and Hart (5) was next used on the assumption that perhaps some substance might be present that caused a fading of the colors produced. This method, while undoubtedly more accurate, especially for examining animal tissue, proved too tedious for the large number of analyses necessary for the proposed work, and it has been quite definitely proved since that time that fading was not the difficulty in the Bergeim method as applied to feed and feces. A modification of the

Kennedy (6) method has proved to be the most rapid and accurate and has been adopted for this study. As applied, it requires the following procedure: 1 gm. of the feed or 0.25 to 0.5 gm. of the dried feces are digested in a 250 cc. Kjeldahl flask with 5 cc. of iron-free sulfuric acid and 2 cc. of perchloric acid. A rack has been prepared so a large number of samples can be handled at the same time. 15 minutes is usually sufficient for complete digestion. The solution is then diluted to 50 cc. and a dilute permanganate solution is added slowly until fully oxidized. It has been found that in many cases considerable oxidation seems necessary even though the perchloric acid has been fully removed. The solution is then transferred with many washings to 100 cc. flasks and diluted to the mark. Aliquot portions are removed to large test-tubes and treated with 5 cc. of 20 per cent sodium cyanate and the color compared at once in a colorimeter with equal aliquots of standards containing varying amounts of iron which have been similarly and simultaneously prepared. These standards are prepared by introducing varying amounts of an iron standard containing 0.1 mg. of iron per cc. in the form of ferric ammonium sulfate.

Complete analysis of each lot of feed, and many times of the feed left in the feeder, together with that of the feces and urine, has been made after each 4 day period from several lots of animals over intervening periods during a 5 months period.

In order to avoid the presentation of an unnecessarily large amount of data, and also to overcome the variations creeping into short time observations, the average analyses of 16 day periods have been calculated and recorded in Table I. It is to be noted, however, that at least four animals were used in each test and a complete analysis carried out at the end of each 4 day period. The even numbers in every case represent the coefficients of utilization of the various constituents, calculated from the amount of these materials found in the feed and feces of a corresponding period. The second column of Table I gives the percentage of iron recovered, calculated from the theoretical amount of iron consumed in the amount of food eaten and the amount actually found in the feces for a corresponding period of time. The coefficients of utilization were calculated by the two methods in the usual way, the results being given in the other columns of the table.

The even numbered samples in every case represent the findings from animals receiving iron only as a normal constituent of the ingested food, the coefficient of utilization being calculated by the Bergeim method on the basis of the normally occurring iron of the feed. The odd numbered samples represent the analysis of a second series of rats fed exactly the same rations, to which have been added varying amounts of ferric oxide, and observed under exactly the same conditions and at the same time as those maintained for the previous lots. It should be stated that these

TABLE I.

Coefficients of Utilization.

Sample No.	Iron recovered.	Standard method, per cent.					Bergeim method, per cent.				
		Protein.	Ether extract.	Ash.	Fiber.	N-free extract.	Protein.	Ether extract.	Ash.	Fiber.	N-free extract.
	per cent										
1*	75.18	65.8	77.25	39.9	31	88.25	57.29	67.63	35.92	28.12	83.04
2	97.18	66.7	74.15	39.6	30.5	89	63.7	79.3	32	34.25	89.19
3*	65.05	68.5	76	41.25	33	90.5	64.77	60.44	29.55	21.67	89.21
4	97.05	64.5	71.15	33.1	37.75	90.5	64.15	73.95	33.1	32.50	90.72
5*	92.4	60.5	74	35.95	33.15	87.35	58.90	77.29	30.56	30.51	86.46
6	97.72	59.85	76.37	35.4	28.9	82.75	62.12	82.88	34.3	40.7	88.08
7*	89.37	52.1	75.55	34.7	25.3	88.1	45.75	71.49	25.5	24.68	85.66
8	97.03	56.1	76.35	32.32	21.32	86.45	55.43	75.33	22.85	22.14	86.10
9*	91.92	47.86	88.1	20.35	19.80	89.71	49.11	91.90	13.84	15.59	83.63
10	98.17	52.45	86.5	19.95	21.2	89.27	50.72	86.06	21.24	17.61	85.74

* Ferric oxide added to the ration.

animals consumed above 5 times as much iron as those eating the basal ration.

Table I presents interesting data requiring some comment. First, it is to be noted that the coefficients of utilization by the two methods correspond surprisingly well for the rations containing no added iron. It has been commonly conceded that iron is excreted largely through the feces, and even if assimilated, it was again excreted into the intestine. If this is true, the Bergeim method might be applied to the rations to which no iron was added and thus be more successful than when first considered, as it would eliminate the criticism of possibly altering the assimila-

tion of food due to possible therapeutic action and also to a very valid criticism that has been observed and will be mentioned later. This would necessitate, however, the use of a ration containing sufficient iron always to meet the needs of the animal and maintaining the animal on this particular ration until the animal's body had established an equilibrium with respect to iron. The analysis of the urine has been made often through the study; but the results are omitted, as it has been found that the amount of iron is so insignificant as not materially to affect our studies. The coefficient of utilization of the ash is obviously merely the measure of the minerals escaping in the feces as compared to that in the urine and is no more variable than might be postulated under the circumstances.

Next, on examination of the data for the animals listed under even numbered samples and consuming iron-supplemented rations, it will be noted that the iron content of the feces of the first two of these does not account for a large percentage of the ingested iron. This presented the greatest obstacle of the study. An analysis of animals failed to reveal any iron storage to explain the loss. The urine was only slightly higher in iron content. Each of the methods tried, notwithstanding all precautions, failed. Finally the analysis of the feed left in the feeders explained the greatest loss, and this incidentally offers the greatest criticism to the method. Despite the fact that the entire ration was finely ground and thoroughly mixed, it was found that the iron oxide had a tendency to separate out, and to what extent the animals were able to effect the separation cannot be stated. To overcome this difficulty the feed was dampened and baked into biscuits, but such a method is not applicable in many cases, and undoubtedly the digestibility of the feed must be so altered. The later determinations were made more nearly successful, so far as the recovery of iron was concerned, by mixing cod liver oil with the ration, and this had a tendency to bind the oxide to the feed. As a further precaution only as much food was provided as eaten, relatively small amounts being left at the end of the feeding periods. Despite all precautions, there was noted invariably some loss of iron in the feces of the animals consuming the iron-supplemented rations. This obstacle would become greater in preparing feed for large animals.

From the above data it would seem that a fairly accurate and at the same time a much more rapid method of determining the coefficients of utilization can be carried out as follows: A uniform mixture of the food to be studied, without the addition of extra iron, is prepared and fed for a period of 2 weeks prior to the time of analysis. Samples of the feces are gathered daily over a considerable period of time. The same are rapidly dried and preserved. 1 gm. samples of feed and 0.5 gm. samples of feces are analyzed for iron by the modified Kennedy method as previously indicated. The other constituents of feed and feces are analyzed by the customary methods and the coefficients of utilization calculated by the original method suggested by Bergeim.

DISCUSSION.

1. Standard utilization studies are accurate only within limits and are so time-consuming as to be of value only in limited studies.

2. The Bergeim method may be applied in utilization studies, as results so obtained are comparable to the older methods, providing indicated precautions are observed.

3. Some iron is usually not recovered when added to the ration.

4. It is exceedingly doubtful whether the iron oxide would be eliminated in a like proportion in all parts of the feces, thereby making possible the sampling of any portion at random during the day.

5. The indications are that most rations contain sufficient iron to apply the Bergeim method without the addition of the ferric oxide. Such procedure should provide more uniform intake and a more evenly distributed iron excretion in the feces.

6. The method suggested, the normal iron content of rations being used, retains the advantage of the original Bergeim method and eliminates the unfavorable steps of that method.

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A COMPARISON OF RAW, PASTEURIZED, EVAPORATED, AND DRIED MILKS AS SOURCES OF CALCIUM AND PHOSPHORUS FOR THE HUMAN SUBJECT.*

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It has been generally recognized that milk serves as an excellent source of calcium and phosphorus in the diet. However, an increasing tendency toward the use of such forms as evaporated, dried, and especially pasteurized instead of raw milk makes it seem of value to determine whether or not the same reliance can be placed upon all forms of milk as sources of the minerals, calcium and phosphorus. Experiments here described were planned to add to the present rather limited knowledge concerning the availability of calcium and phosphorus from various forms of milk, fresh raw milk being used as a standard and both children and adults as subjects.

LITERATURE.

A search of the literature fails to reveal many metabolism experiments with the human subject for the study of the problem at hand. Furthermore, almost none of the experiments had a definite standard for comparison.

Willard and Blunt (1) have reviewed the more important studies made with both human and animal subjects; namely, the work of Washburn and Jones (2), Magee and Harvey (3), Daniels and Loughlin (4), and Daniels and Stearns (5). These investigations point to the superiority of raw, evaporated, condensed, and quickly boiled milks over pasteurized milk. Willard and Blunt (1) made an extensive balance study, using pasteurized and evaporated milks. The children used as subjects all showed better retention of calcium and phosphorus when evaporated rather than pasteurized milk was used. Increased retention on evaporated milk was shown by two of their four adult subjects.

* Contribution No. 6, Department of Home Economics.

Ladd, Evarts, and Franks (6) made a study of the relative efficiency of certified and pasteurized milk for infant feeding by following the gains in weight made by a large number of subjects. They found that the certified milk allowed for better gains than the pasteurized, even when orange juice and cod liver oil were added. They raise the question whether the superiority of the certified milk is due merely to the fact that it is raw or to the better balanced feeding of the cows.

Differences in availability of the minerals in the food seems even more important in the light of the report of Hart, Tourtellotte, and Heyl (7) whose adult subject showed no increase in calcium retention as the result of irradiation or as the result of the administration of cod liver oil.

EXPERIMENTAL.

Calcium and phosphorus balance experiments were carried out in three series, both children (Series 1, conducted in Topeka) and adults (Series 2 and 3, conducted in Manhattan) being used as subjects. The same general plan was used throughout, changes being made only where experience indicated possible improvements. In all cases every effort was made to have milk the only variable in the experiment. Simple diets were planned, adequate in protein (8) and calories. Milk was made to furnish as much of the total calcium as possible. Milk also furnished much of the phosphorus, but its composition is such that it could not provide equally large proportions of both elements. In order that differences might show, it seemed essential to keep the total intake of calcium and phosphorus near the minimum required for maintenance in the adults (8) and below the amount required for optimum storage in the children.

The children were given about 625 gm. of fresh milk daily and equivalent amounts of dried milk, this being below the figure for optimum storage suggested by Sherman and Hawley (9). Fresh milk furnished not less than 75 per cent and dried milk not less than 73 per cent of the total calcium. Almost half the phosphorus was supplied by the milk.

The adults in Series 2 received 222 gm. of fresh milk daily, with calculated equivalent amounts of the other forms during the various periods. Analyses later showed that the subjects received about 0.56 gm. of calcium per day. The milk furnished 47 to 52 per cent of the total calcium but only about one-fourth of the total phosphorus. In Series 3 the fresh milk was increased to 260 gm. per day, but the diet was so changed as to bring the total calcium down

to 0.50 gm. per day, which is nearer the minimum of 0.45 gm. set by Sherman (8). The milk furnished 68 per cent of the total calcium and 38 per cent of the total phosphorus.

As much as possible foods were chosen which could be purchased in quantity to last through an experiment so that the composition would not vary.

A standard brand of dried milk made by the spray process and also a popular brand of evaporated (*i.e.* unsweetened condensed) milk were chosen as examples of these products now in common use. These same commercial brands were used in all experiments. In Topeka the fresh milk was obtained from a commercial dairy marketing a product of very high grade. The fresh milk for Series 2 and 3 was obtained from the College Dairy. For Series 2 it happened that milk was available from two cows which had been kept in the dairy barn away from the sunlight for some months before coming fresh. The cows had been in the barn 5 months at the time the milk (called Fresh Milk B) was used for the experiments. The cows producing this milk were of the college herd and were receiving the same rations as the animals in the remainder of the herd from which the fresh milk for the experiments was obtained. For Series 3 milk pasteurized by the "hold" process was obtained from the College Dairy. It was pasteurized in the morning and used at noon. It was of exactly the same origin as the fresh milk used in the same series.

All experiments were divided into 3 day periods. Foods were weighed and records kept of daily consumption of subjects. Food samples of approximately the same size were taken daily, weighed, and dried or, in the case of liquids, preserved with formaldehyde for analysis. All samples of urine and feces were collected, carmine being used to mark the feces of the various periods.

The McCrudden (10) method of analysis was used for calcium determinations of both food and feces for all series. Dry samples were ashed in an electric muffle furnace, and liquid samples with nitric acid.

For samples of Series 1 and 2 the phosphorus determinations were made by the gravimetric method of double precipitation, first as ammonium phosphomolybdate and then as magnesium ammonium phosphate. A volumetric method recommended by the Association of Agricultural Chemists (11) was used for deter-

mination of phosphorus in samples for Series 3. Magnesium nitrate was used for ashing. The methods used were tested for accuracy on samples of known composition. Analyses were made with the cooperation of the chemists of the Agricultural Experiment Station and the methods were those used by them in routine analyses. All analyses were made in duplicate.

Since the content of calcium and phosphorus varies within wide limits for most foods, analyses were made of all foods used, no figures being accepted from previously compiled tables (Table I).

TABLE I.

Food Analyses (Percentage of Edible Portion).

	Series 1.		Series 2.				Series 3.	
			First six periods.		Last two periods.			
	Ca	P	Ca	P	Ca	P	Ca	P
Fresh milk	0.124	0.024	0.127	0.023	0.127	0.023	0.134	0.105
Fresh Milk B					0.125	0.025		
Pasteurized milk							0.134	0.104
Dried milk	0.915	0.181	0.976	0.176			0.934	0.768
Evaporated milk			0.256	0.046			0.283	0.210
Apple	0.033	0.004						
Bread	0.012	0.020	0.052	0.019	0.051	0.026	0.050	0.092
Butter	0.017	0.004	0.028	0.004	0.030	0.004		
Cocoa	0.032	0.184						
Rolled oats	0.136	0.106						
Orange juice	0.012	0.006	0.014	0.052	0.018	0.005	0.008	0.014
Potato	0.022	0.016	0.032	0.018	0.026	0.014	0.006	0.042
Prunes	0.028	0.031	0.078	0.016	0.082	0.025	0.055	0.095
Lean ground beef			0.030	0.051	0.018	0.050	0.016	0.223

Series 1 (Started in October, 1924).—Three girls and two boys, 7 to 12 years of age, served as subjects for this series. The feeding experiments were conducted in the Topeka Home of the Kansas Children's Home Society where necessary facilities for the work were kindly provided. The children were examined by a physician, a child specialist, and were pronounced suitable for the investigation. The experiment was divided into the following periods: Period I, fresh milk; Period II, fresh milk; Period III, dried milk; Period IV, dried milk; Period V, fresh milk.

The regular hours of the institution were maintained for the

children used as subjects. Time spent in the open air, including walks to and from the public school a few blocks distant, remained practically uniform throughout the experiment. During the entire time only 2 days were cloudy, sunny fall weather typical of the region prevailing during the remainder of the time. The

TABLE II.

Series 1.

Eunice, female, age 12 yrs., weight 38.2 to 41.1 kilos.

Period No.	I	II	III	IV	V
Average food intake for each period, in gm. per day.					
Fresh milk	662	625			625
Dried "			82	82	
Apple, E. P.*	208	200	200	217	200
Bread	318	299	384	325	299
Butter	90	110	110	150	93
Cocoa	5	7	7	7	4
Oats	40	45	45	45	45
Orange juice	57	65	65	65	65
Potato, E. P.	198	250	250	250	250
Prunes, "	25	50	50	50	50
Sugar	27	35	27	20	22
Calories	2759	2919	3093	3232	2724
Protein	66	66	74	69	65
Calcium	1.051	1.030	1.018	1.023	1.026
Phosphorus	0.657	0.684	0.711	0.692	0.672
Ca and P balances in gm. per day.					
Ca output	0.433	0.699	0.785	0.626	0.572
" balance	+0.618	+0.331	+0.233	+0.397	+0.454
P output	0.254	0.272	0.374	0.352	0.354
" balance	+0.403	+0.412	+0.337	+0.340	+0.318

*E. P., edible portion.

nutritive condition of the children remained good throughout and each subject showed a satisfactory gain in weight.

The diet contained, daily, 625 gm. of fresh milk or its calculated equivalent in dried milk. Other foods were apples, cocoa, orange juice, prunes, potatoes, rolled oats, sugar, bread, and butter. The last two items were given in amounts desired but the other foods were kept nearly constant (Table II).

Series 2 (*Started in February, 1926*).—Four healthy young women who were instructors and graduate students served as subjects for the second series of experiments. The periods of the

TABLE III.

Series 2.

V. C., female, weight 63 kilos.

Period No.....	I	II	III	IV	V	VI	VII	VIII
Average food intake for each period, in gm. per day.								
Dried milk..	30	30						
Fresh " ..			222	222				
Evaporated milk.....					100	100		
Fresh Milk B.....							222	222
Bread, white.....	250	250	250	250	250	250	230	230
Butter.....	100	100	100	100	100	100	70	70
Orange juice.....	200	200	200	200	200	200	200	200
Potato, E. P.*....	120	120	120	120	120	120	120	120
Prunes, E. P.	50	50	50	50	50	50	50	50
Beef, lean..	80	80	80	80	80	80	80	80
Sugar.....	90	90	90	90	90	90	70	70
Calories....	2391	2391	2392	2392	2406	2406	2029	2029
Protein.....	52	52	52	52	55	55	50	50
Calcium....	0.580	0.580	0.568	0.568	0.543	0.543	0.538	0.538
Phosphorus.	0.373	0.373	0.371	0.371	0.360	0.360	0.393	0.393
Ca and P balances in gm. per day.								
Ca output..	0.686	0.680	0.580	0.581	0.536	0.545	0.650	0.655
" balance.	−0.106	−0.100	−0.012	−0.013	+0.007	−0.002	−0.112	−0.117
P output...	0.388	0.383	0.371	0.369	0.361	0.369	0.534	0.526
" balance..	−0.015	−0.010	0.000	+0.002	−0.001	−0.009	−0.141	−0.133

* E. P., edible portion.

experiment were as follows: Period I, dried milk; Period II, dried milk; Period III, fresh milk; Period IV, fresh milk; Period V, evaporated milk; Period VI, evaporated milk; Period VII, fresh milk B; Period VIII, fresh milk B.

A diet was planned to follow closely that of the children, the calcium being reduced to the point where differences might be expected to show. The diet contained white bread, butter, orange juice, potatoes, prunes, lean ground beef, and sugar. Body weights of subjects were kept constant by adjusting the amounts of butter and sugar in the diets. Subjects remained indoors as

TABLE IV.
Series 3.

I. B., female, weight 58.2 kilos.

Period No.	I	II	III	IV	V	VI
Average food intake for each period, in gm. per day.						
Pasteurized milk	260	260				
Raw milk			260	260		
Dried milk					34	34
Bread	200	200	200	200	200	200
Butter fat	90	90	90	80	80	80
Orange juice	200	200	200	200	200	200
Potato, E. P.*	120	120	120	120	120	120
Prunes, "	35	35	35	35	35	35
Beef	80	80	80	80	80	80
Sugar	80	80	80	70	70	70
Calories	2244	2244	2244	2034	2027	2027
Protein	48	48	48	48	48	48
Calcium	0.503	0.503	0.503	0.503	0.472	0.472
Phosphorus	0.711	0.711	0.711	0.711	0.702	0.702
Ca and P balances in gm. per day.						
Ca output	0.632	0.619	0.525	0.453	0.530	0.532
" balance	−0.129	−0.116	−0.022	+0.050	−0.058	−0.060
P output	0.813	1.057	0.692	0.800	0.693	0.766
" balance	−0.102	−0.346	+0.019	−0.089	+0.009	−0.064

* E. P., edible portion.

much as possible, so that the factor of exposure to sunlight was almost eliminated (Table III).

Series 3 (Started in February, 1928).—Four healthy young women who were instructors and graduate students served as subjects for the third series of experiments, which was conducted in the late winter as was the second series. The general plan of the experiments was much the same. The periods were: Period I,

pasteurized milk; Period II, pasteurized milk; Period III, fresh milk; Period IV, fresh milk; Period V, evaporated milk for two subjects and dried milk for two subjects; Period VI, evaporated milk for two subjects and dried milk for two subjects.

Profiting by the previous experiments, we made a few changes. In the first place, the diet was so planned that a larger per cent of the total calcium would be furnished by the milk. The total intake of calcium was somewhat lower than in Series 2, bringing

TABLE V.

Series 1. Calcium and Phosphorus Balances in Gm. per Day (Average of Periods Used).

Subject.	Kind of milk.	Calcium.				Phosphorus.			
		Intake.	Output.	Balance.	Balance per kg.	Intake.	Output.	Balance.	Balance per kg.
Eunice, 12 yrs., 40 kilos.	Fresh.	1.032	0.568	0.464	0.012	0.671	0.293	0.378	0.009
	Dried.	1.025	0.706	0.319	0.008	0.702	0.363	0.339	0.008
Jack, 9 yrs. 8 mos., 32 kilos.	Fresh.	1.066	0.761	0.305	0.010	0.654	0.391	0.263	0.008
	Dried.	0.939	0.732	0.207	0.007	0.628	0.473	0.155	0.005
Lottie, 8 yrs. 3 mos., 26 kilos.	Fresh.	1.043	0.493	0.550	0.021	0.682	0.372	0.310	0.012
	Dried.	0.991	0.596	0.395	0.015	0.666	0.425	0.241	0.009
Nellie, 10 yrs. 6 mos., 28.3 kilos.	Fresh.	1.037	0.325	0.712	0.025	0.652	0.262	0.390	0.014
	Dried.	0.978	0.603	0.375	0.013	0.639	0.357	0.282	0.010
Wayne, 7 yrs. 4 mos.	Fresh.	1.046	0.767	0.279	0.011	0.684	0.402	0.282	0.012
	Dried.	1.002	0.828	0.174	0.007	0.664	0.388	0.276	0.011

the figure nearer the minimum requirement of 0.45 gm. suggested by Sherman (8). Also, more rigid rules were set up for the consumption of food by the subjects. All subjects ate the same foods at the same time of day. All milk was consumed, without heating, as a beverage at the noon meal. The pasteurized and raw milks, which came from the College Dairy, were never older than of the milking of the previous night.

All subjects remained indoors as much as possible, although there was little sunny weather during the time of the experiment.

TABLE VI.

Series 2. Calcium and Phosphorus Balances in Gm. per Period (Average of Periods Used).

Subject.	Kind of milk.	Calcium.				Phosphorus.			
		Intake.	Output.	Balance.	Balance per kg.	Intake.	Output.	Balance.	Balance per kg.
M. D., weight 56 kilos.	Fresh.	1.730	1.487	+0.243	+0.004	1.121	1.034	+0.087	+0.002
	Dried.	1.773	1.748	+0.025	0.000	1.130	1.145	−0.015	0.000
	Evaporated.	1.649	1.589	+0.060	+0.001	1.088	1.029	+0.059	+0.001
R. T., weight 69.5 kilos.	Fresh.	1.704	1.416	+0.288	+0.004	1.113	1.053	+0.060	+0.001
	Dried.	1.740	1.583	+0.157	+0.002	1.119	1.133	−0.014	0.000
	Evaporated.	1.629	1.478	+0.151	+0.002	1.080	1.070	+0.010	0.000
V. C., weight 63 kilos.	Fresh.	1.704	1.742	−0.038	−0.001	1.013	1.010	+0.003	0.000
	Dried.	1.740	2.049	−0.309	−0.005	1.119	1.157	−0.038	−0.001
	Evaporated.	1.629	1.622	+0.007	0.000	1.080	1.095	−0.015	0.000
	Fresh Milk B.	1.614	1.958	−0.344	−0.005	1.079	1.590	−0.511	−0.008
M. K., weight 53.5 kilos.	Fresh.	1.647	1.517	+0.130	+0.002	1.083	1.058	+0.025	0.000
	Dried.	1.689	1.778	−0.089	−0.002	1.091	1.085	+0.006	0.000
	Evaporated.	1.587	1.422	+0.165	+0.003	1.056	0.947	+0.109	+0.002
	Fresh Milk B.	1.614	1.647	−0.033	−0.001	1.179	1.548	−0.369	−0.007

TABLE VII.

Series 3. Calcium and Phosphorus Balances in Gm. per Period (Average of Periods Used).

Subject.	Kind of milk.	Calcium.				Phosphorus.			
		Intake.	Output.	Balance.	Balance per kg.	Intake.	Output.	Balance.	Balance per kg.
E. L., weight 53.6 kilos.	Fresh.	1.474	1.234	+0.240	+0.004	2.064	2.050	+0.014	0.000
	Dried.	1.382	1.653	−0.271	−0.005	2.038	2.048	−0.010	0.000
	Pasteurized.	1.509	2.236	−0.727	−0.014	2.133	2.551	−0.418	−0.008
I. B., weight 58.2 kilos.	Fresh.	1.509	1.468	+0.041	+0.001	2.133	2.238	−0.105	−0.002
	Dried.	1.417	1.593	−0.176	−0.003	2.107	2.188	−0.081	−0.001
	Pasteurized.	1.509	1.876	−0.367	−0.006	2.133	2.804	−0.671	−0.012
A. L., weight 68 kilos.	Fresh.	1.509	1.881	−0.372	−0.005	2.133	2.032	+0.101	+0.001
	Evaporated.	1.525	1.556	−0.031	0.000	2.110	2.092	+0.018	0.000
	Pasteurized.	1.509	1.883	−0.374	−0.006	2.110	2.092	+0.018	0.000
M. K., weight 56.3 kilos.	Fresh.	1.509	1.550	−0.041	−0.001	2.133	2.236	−0.103	−0.002
	Evaporated.	1.525	1.318	+0.207	+0.004	2.110	1.835	+0.275	+0.005
	Pasteurized.	1.509	1.803	−0.294	−0.005	2.133	2.348	−0.215	−0.004

One subject only had received cod liver oil and that had been some weeks before the experiment started.

The foods selected for the diet were white bread, potatoes, ground lean round of beef, prunes, orange juice, sugar, butter fat, and milk. Quantities of butter fat and sugar were adjusted so that the subjects remained constant in weight (Table IV).

TABLE VIII.

Comparisons of Balances of Calcium per Kilo per Period of Subjects on Various Kinds of Milk.

The balance of each subject on raw milk was taken as his or her standard or zero for the comparisons tabulated below.

	Pasteurized.	Dried.	Evaporated.	Fresh milk B.
Series 1.				
Eunice		−0.012		
Jack		−0.009		
Lottie		−0.018		
Nellie		−0.036		
Wayne		−0.012		
Series 2.				
M. D		−0.004	−0.003	
R. T		−0.002	−0.002	
V. C		−0.004	+0.001	−0.004
M. K		−0.004	+0.001	−0.003
Series 3.				
I. B	−0.007	−0.004		
E. L	−0.018	−0.009		
M. K	−0.004		+0.005	
A. L	−0.001		+0.005	
Averages.				
Series 1 (children)		−0.017		
Series 2 (adults)		−0.004	−0.001	−0.004
Series 3 (adults)	−0.008	−0.007	+0.005	
For all adults	−0.008	−0.005	+0.001	−0.004

DISCUSSION.

The experiment with children showed good storage of calcium from fresh milk not only at the beginning of the experiment but at the end also when the fresh milk period was repeated. During

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the dried milk periods the diets contained almost as much calcium and a little more phosphorus than during the fresh milk periods. However, figures show the calcium retention of the various children to have averaged only 53 to 71 per cent as much as during their fresh milk periods, although the dried milk periods furnished an average of 94.5 per cent as much calcium as did the fresh milk periods. Likewise the phosphorus retention was somewhat lower. It is thus evident that the child retains more calcium when it is supplied in fresh milk than when furnished in equal amounts by dried milk, other factors remaining unchanged (Table V).

The adult subjects in Series 2 and in Series 3 were at all times receiving close to the minimum amounts of calcium and phosphorus, sometimes showing negative balances and sometimes positive. (Tables VI and VII). Calculations have been made to show the calcium and phosphorus balances of each subject in gm. per kilo of body weight per period, averaged for like periods. The 3 day period was used, rather than the day, as the figures are necessarily rather small. Comparisons were then made, subject by subject, in each case the balance of a subject on fresh milk being taken as zero and the balances on other milks being listed as higher and lower or plus and minus in comparison with this fresh milk figure (Table VIII).

As with the children, dried milk gave for all subjects a lower calcium balance than did the fresh milk. Pasteurized milk, used in Series 3, gave a lower calcium balance than did fresh milk. On the other hand evaporated milk, used in both Series 2 and 3, gave a higher calcium balance for four of the six subjects. In Series 3, the most carefully controlled experiments, the subjects showed distinctly better calcium balances on evaporated milk than on fresh milk. In Series 2, Fresh Milk B, secured from cows kept in the barn for 5 months, gave far lower calcium balances than did the herd fresh milk. Utilization of calcium from this milk was only about as good as the utilization of calcium from the dried milk.

Since the amount of milk used furnished smaller proportions of the phosphorus of the diet, the results on phosphorus are necessarily less convincing. They follow in general the results for calcium.

SUMMARY AND CONCLUSIONS.

Metabolism experiments have been conducted with children and adults as subjects in an effort to learn whether or not the calcium and phosphorus in various forms of milk are equally available for human nutrition.

Results of experiments with five children make it evident that the child retains more calcium when it is supplied in fresh milk than when it is furnished in equal amounts by dried milk, other factors remaining unchanged.

All adult subjects showed more favorable calcium balances when the fresh milk was the source of supply rather than dried milk. Pasteurized milk also gave less favorable calcium balances than did fresh milk. Further, the milk from cows kept in the barn (Fresh Milk B) gave less favorable calcium balances than did fresh milk.

On the other hand adult subjects using evaporated milk showed balances at least as good as when fresh milk was used.

In general phosphorus balances followed the trend of the calcium balance figures.

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A COMPARISON OF pH DETERMINATIONS AS OBTAINED BY MEANS OF HYDROGEN ELECTRODE AND COLORIMETRIC METHODS.

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The wide application of studies of the hydrogen ion concentration of blood and other body fluids suggests the importance of a simple method for these determinations. The hydrogen electrode method, which is generally taken as standard, requires skill and a considerable amount of time and of blood. To overcome these disadvantages colorimetric methods have been devised, based on the comparison of colors given by an indicator when added to an unknown solution and to standard buffer solutions. Errors are encountered because the color of the indicator is influenced by proteins and salts as well as by the pH of the solutions. Cullen (1922, *a*) diluted serum twenty times with 0.9 per cent sodium chloride solution and concluded that the differences between the colorimetric and hydrogen electrode readings were sufficiently constant to employ a correction in order to convert colorimetric readings at 20° to the electrometric values at 38°. Hastings and Sendroy (1924) reported that this correction became zero when they compared colorimetric readings at 38° with the electrometric readings at 38°. But Bennett (1925–26) and Austin, Stadie, and Robinson (1925) compared the Cullen and the Hastings procedures with the electrometric (and gasometric) method and found the corrections to vary considerably.

A dialysis method for the determination of pH was originated by Levy, Rowntree, and Marriott (1915) and improved by Dale and Evans (1920–21). In this method blood is dialyzed and the indicator is added to the dialysate. The protein is practically completely absent from the solution tested but salts are present and

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will affect the colorimetric readings. It was thought desirable to study the dialysis method with the hope that the differences between the colorimetric and electrometric readings would be constant. Determinations were run by each of these colorimetric methods (the dialysis, the Hastings, and the Cullen methods) on the same blood and the results were compared with the value obtained by means of the hydrogen electrode.

EXPERIMENTAL.

Serum was used for the electrometric, the Cullen, and the Hastings methods. The whole blood for the dialysis method was obtained by allowing about 15 cc. to run into a tube containing oxalate, in the middle of the blood collection. The portion collected in this manner was assumed to be similar to the whole sample. In order to test the validity of this assumption several determinations were made by dialyzing the serum as well as the whole blood. The results in all cases were similar.

The blood used in these experiments was obtained from dogs. The femoral artery was exposed under cocaine, a cannula placed in the artery, and blood collected under oil in hard glass centrifuge tubes. After a sample was taken, the animal was allowed to bleed enough to make the total hemorrhage $3\frac{1}{2}$ to 4 per cent of the body weight and then a second sample was taken either immediately or after a period of 5 to 15 minutes. The dogs were rebled on the following day if they survived. At no time during the collection or handling of the blood was it allowed to come in contact with the air. Centrifuging was done under solidified paraffin and the serum drawn into hard glass tonometers over clean mercury as suggested by Austin, Cullen, *et al.* (1922).

The electrometric determinations were made at 38° with standard apparatus. The serum was placed in a Clark-Cullen electrode vessel and allowed to come to equilibrium with a carbon dioxide-hydrogen mixture (Cullen, 1922, *b*). Fresh portions of serum were introduced, the same gas mixture being kept until constant E.M.F. readings were obtained.

The Cullen colorimetric readings were made as described by Cullen (1922, *a*) except that a definitely measured amount of 0.02 per cent phenol red (0.1 cc.) was used instead of the 5 drops suggested by him. The sodium chloride solution was brought to pH

7.4 by passing a current of CO_2-free air through it (Earle and Cullen, 1928). The pH of the salt solution does not remain at 7.4 when put in the tubes under oil due to the absorption of traces of CO_2 into the unbuffered solution. It is, nevertheless, probably superior to the neutralized solution originally suggested. The Hastings readings were made in the same manner as the Cullen except that they were read with standards and unknowns at 38° instead of at 20°. The comparison tubes were made up from phosphate buffers, 0.05 pH apart, checked electrometrically at 38°.

The dialysis method was carried out as suggested by Dale and Evans (1920–21) with some modifications. Salt solution which had been brought to pH 7.4 as in the Cullen method was run into a small test-tube under oil. A collodion bag containing a few drops of oil was suspended in about 1.5 cc. of salt solution. Approximately 1 cc. of blood was then introduced into the bag under oil and allowed to dialyze at the desired temperature. It was not found necessary to stopper the tubes in which the dialysis was carried on if the blood and dialysate were kept under oil and stirring was minimized. This fact was ascertained by running a series with and without stoppers. Similar results were obtained in both cases. After dialysis for 30 minutes the sack containing the blood was removed and 0.1 cc. of 0.02 per cent cresol red solution was added. This was then compared with freshly prepared buffer standards 0.05 pH apart, containing the same amount of indicator. The temperatures of the dialysate and the standard were kept the same by means of a water bath.[1] The length of time for dialysis was arrived at by dialyzing portions of blood for varying lengths of time at 20°. It was found that although the samples dialyzed 15, 30, 45, or 60 minutes usually gave the same pH readings, occasionally a sample dialyzed for 15 minutes would give a reading lower than those dialyzed longer.

The collodion bags used were made from a 12 to 15 per cent solution of parlodion in equal parts of alcohol and ether. The solution was poured into test-tubes of appropriate size, drained for 2 minutes, and 70 per cent alcohol added (Gates, 1922). After 2 or 3 minutes the alcohol was poured off and the sacks loosened with distilled water. The sacks were then placed in a large vol-

[1] In the early experiments, certain readings were made at the prevailing room temperature as shown in Table I.

TABLE I.

Comparison of Results of Electrometric and Colorimetric pH Determinations.

Dog No.	Date.	Blood No.	Hydrogen electrode pH_e 38°.	Dialysis methods.			Dilution methods.		
				Dialysis at room temperature.		Dialysis at 38°. $pH_e38° - pH_e38°$.	Cullen.		Hastings.
				Temperature.	$pH_e - pH_e38°$.		Temperature.	$pH_e - pH_e38°$.	$pH_e38° - pH_e38°$.
	1927			°C.			°C.		
13	Apr. 5	1	7.38	23	0.17				
16	" 15	1	7.42	19	0.28	0.33			
		2	7.62	19	0.18	0.03			
17	" 21	1	7.45	23	0.10				
		2	7.55	23	0.10				
	" 22	1	7.41	23	0.24				
13	June 16	1	7.40				22	0.34	0.10
		2	7.40				22	0.34	0.12
	" 17	1	7.41				21	0.18	0.04
		3	7.36				21	0.39	0.11
	" 22	1	7.35				24	0.40	0.10
		2	7.46				24	0.34	0.09
		3	7.27				24	0.48	0.18
	" 23	2	7.40				20	0.25	0.10
		3	7.44				20	0.36	0.06
26	" 29	1	7.32				20	0.45	0.13
		2	7.22				20	0.50	0.18
	" 30	1	7.32	25	0.28		20	0.22	0.06
		2	7.33	25	0.17		20	0.22	0.05
		3	7.43	25	0.42		20	0.42	0.27
		4	7.08	25	0.37				
		5	7.10	25	0.45		20	0.17	0.05
27	July 14	1	7.35	28	0.17				
		2	7.36	28	0.19				
	" 16	1	7.33	28	0.11		20	0.27	0.12
28	" 19	1	7.25	26	0.23		20	0.50	0.18
		2	7.32	26	0.23		20	0.23	0.0
	" 22	1	7.30	27	0.15		20	0.25	0.05
		2	7.30	27	0.35		20	0.25	0.05
29	" 26	1	7.27	27	0.13		20	0.45	0.13
30	Aug. 17	1	7.33	20	0.12		20	0.42	0.15
		2	7.35	20	0.10		20	0.40	0.10
		3	7.30	20	0.13		20	0.42	0.15

TABLE I—*Concluded.*

Dog No.	Date.	Blood No.	Hydrogen electrode $pH_e38°$.	Dialysis methods.			Dilution methods.		
				Dialysis at room temperature.		Dialysis at 38°. $pH_e38°-pH_e38°$.	Cullen.		Hastings.
				Temperature.	$pH_e-pH_e38°$.		Temperature.	$pH_e-pH_e38°$.	$pH_e38°-pH_e38°$.
	1927			°C.			°C.		
30	Aug. 18	1	7.32	20	0.28				
		2	7.44	20	0.36				
		3	7.00	20	0.35				
13	Sept. 14	1	7.37	20	0.13	−0.02	20	0.35	0.03
		2	7.31	20	0.24	0.04	20	0.36	0.04
	" 15	1	7.31	20	0.34	0.34	20	0.21	0.04
		2	7.53	20	0.32	0.32	20	0.32	0.05
31	" 21	1	7.35	20	0.25	0.10	20	0.37	0.10
		2	7.42	20	0.38	0.13	20	0.36	0.08
	" 22	1	7.38	20	0.27	0.04	20	0.27	0.02
		2	7.67	20	0.28	−0.02	20	0.33+	0.08
32	Oct. 18	1	7.25	20	0.30	0.15			
		2	7.44	20	0.26	0.11			
33	Nov. 10	1	7.33	20	0.32	0.10			
		2	7.32	20	0.33	0.11			
	" 11	1	7.37	20	0.28	0.08	20	0.21	0.00
		2	7.52	20	0.38	0.13	20	0.23	0.06
34	" 18	1	7.39	20	0.21		20	0.28	0.04
		2	7.59	20	0.16		20	0.34	0.08
35	" 28	1	7.39	20	0.39	0.28	20	0.26	0.00
		2	7.42	20	0.41	0.28	20	0.31	0.05

pH, 38° indicates the pH determined at 38° by means of the electrometric (hydrogen electrode) method. pH_e indicates the pH determined by means of one of the colorimetric methods at the temperature shown. The corrections for the colorimetric methods are given as the differences between the colorimetric pH and the electrometric pH values. Only the differences are given as the colorimetric readings may be readily calculated by adding the differences shown in the table. Blood 1 was taken immediately before a severe hemorrhage. The others were taken at various times after hemorrhage.

ume of 0.9 per cent salt solution, and allowed to stand for 2 or 3 days before being used. They were washed well with salt solution before being used and were never used more than once. As parlodion develops some acidity on standing in water, solutions were made up from several samples to see whether those which were most acid would lower the pH readings obtained by the dialysis method. The readings that were made from the sacks prepared from the acid parlodion compared favorably with those from the sacks made of parlodion which was neutral in reaction. In accordance with the results obtained by Levy, Rowntree, and Marriott (1915) variations in thickness of sacks made no difference in readings. A comparison of the readings obtained by the methods studied is shown in Table I.

All determinations were done in duplicate except in the case of the dialysis method where a series of five separate determinations was made for each reading. All colorimetric tubes were brought to the desired temperature by means of a water bath.[1]

DISCUSSION.

Cullen (1922, *a*) found a correction for his method of 0.35 pH for dog blood, and 0.23 pH for human blood to make colorimetric readings at 20° correspond to electrometric readings at 38°. Hastings and Sendroy (1924) reported a very good agreement between the colorimetric and electrometric readings when both were made at 38°. Our data show variations in the Cullen correction from 0.13 pH to 0.41 pH and variations in the Hastings correction from 0.00 to 0.18 pH, so that even the variations in the Hastings correction are sufficiently great to make the Hastings method too rough for accurate work.[2]

The data presented show the correction of the dialysis method to be inconstant. As in the case of the dilution method the correction diminishes as the temperature is raised from 20° to 38°. The differences between the readings at 20° and those at 38° are not constant so that it is not possible to read at one temperature

[2] Incidentally, these results confirm the observations of Miss Bennett that the Cullen correction becomes lower than normal the day after hemorrhage. In the present study the samples after hemorrhage were taken too soon to permit any appreciable variation to take place, though there is usually a slight rise in the correction.

and correct to another. The dialysates which had been read at 20° were then placed in a water bath at 38° and, after being allowed to reach the temperature of the bath, were read again at the higher temperature. The results were so variable that no conclusions could be drawn from them.

Evans (1921) found that the difference between the results of the dialysis method and the electrometric or gasometric method was about 0.20. This correction factor was obtained from studies on 0.02 M $NaHCO_3$ at varying CO_2 tensions. The electrometric and colorimetric determinations were both made at 20°. The individual corrections varied from 0.12 pH to 0.27 pH and the average of five experiments was just below 0.20 pH. For use with blood the method was checked on two samples in which the corrections were 0.15 and 0.20. Evans' original idea that the electrometric values were too low was subsequently shown to be incorrect. Bayliss, Kerridge, and Verney (1926) compared the dialysis, glass electrode, and hydrogen electrode methods and found very good agreement between the two electrometric methods. Comparing the dialysis and the glass electrode methods they showed that the correction necessary to make the colorimetric readings correspond to the electrometric readings varied from −0.25 pH to +0.25 pH. These variations are not only much greater than ours, but they differ also in that our colorimetric readings were practically never lower than electrometric readings. We have only twice observed a small negative correction. As the average of their variations is about zero they draw the conclusion that the dialysis method is accurate to 0.02 pH.

Although there are data in the literature showing variations in the corrections of the colorimetric readings they have aroused little concern on the part of those using the colorimetric methods. Conclusions have been drawn from colorimetric data without consideration of the possibility of variations in the correction. In Table II there are collected, from various sources, data which not only indicate the variability of colorimetric corrections but also point out that the corrections are variable in other species as well as the dog.

Of the colorimetric methods studied, the Hastings and Sendroy modification of Cullen's dilution method yielded the smallest corrections and minimum variations in the corrections. It would

TABLE II.

Variations in Colorimetric Corrections.

Author.	Source.	Dilution method.		Dialysis method.	
		Cullen. $pH_c20°-pH_e38°$.	Hastings. $pH_c38°-pH_e38°$.	$pH_c20°-pH_e38°$.	$pH_c38°-pH_e38°$.
Conway-Verney and Bayliss (1923–24).	Sheep.				0.02–0.05 (1)*
Chambers and Kleinschmidt (1923).	Human (normal).			0.09–0.20 (12)	
	(Clinical cases.)			0.10–0.31 (31)	
Hastings and Sendroy (1924).	†		0.02–0.04 (10)		
Marrack and Smith (1924).	Human (clinical cases).	0.20–0.26 (19)			
Austin, Stadie, and Robinson (1925).	Dog.	0.20–0.47 (21)	0.00–0.17 (28)		
	Sheep.	0.06–0.27 (7)	0.00–0.06 (11)		
	Rat.	0.16–0.18 (2)	0.02–0.06 (2)		
	Human (clinical cases).	0.19–0.51 (8)	0.01–0.20 (11)		
Cullen, Keeler, and Robinson (1925).	" "	0.24–0.35 (18)	0.01–0.12 (9)*		
Earle and Cullen (1928).	Human (normal).		0.06–0.09 (11)*		
Bennett (1926).	Dog.	0.21–0.46 (34)	0.15–0.22 (5)		
Present report.	"	0.17–0.50 (36)	0.00–0.18 (38)	0.00–0.41 (44)	0.02–0.32 (16)

Figures in parentheses indicate number of different bloods studied.

* Both colorimetric and electrometric determinations run at the same temperature but other than 38°, usually 20°.

† Bloods studied were horse (5), dog (1), rat (1), normal human (2).

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appear that the procedure is probably suitable for much work where errors of 0.10 pH are not objectionable, but for accurate studies where small variations are of importance it is obviously untrustworthy.

After this paper was sent to the publishers a statistical study on the same subject appeared by Myers and Muntwyler (1928). They report comparisons of results obtained by using the Cullen, Hastings, and hydrogen electrode methods on 103 samples of human plasma from restricted groups of patients and ten samples of dog plasma. They find that, when a constant Cullen correction of 0.22 pH is subtracted to correct the colorimetric readings obtained by Cullen's method, 59 per cent of the colorimetric values were within ± 0.02 pH, 74 per cent within ± 0.03 pH, and 85 per cent within ± 0.04 pH of the electrometric values. The remaining 15 per cent of the colorimetric readings differed from the electrometric readings by as little as 0.14 and as much as 0.30 pH so that the use of the constant Cullen correction with this group would yield results having considerable error. The data obtained by the Hastings method showed similar variations, no correction being used. The conclusion is drawn that "very good agreement" was obtained. Such a conclusion agrees with the observation of Bayliss, Kerridge, and Verney that the average of the differences between the Dale and Evans method and the glass electrode method equalled zero, though the differences ranged from $+0.25$ to -0.25 pH.

These conclusions are in such sharp contrast with those drawn by Bennett, Austin, Stadie, and Robinson, and the present writer that some explanation is necessary to clarify the situation. There is no indication that there are serious errors in the data of any of these investigations. The different conclusions are probably due to differences in the points of view of the investigators.

The original work of Cullen as well as the later contributions of Hastings, Bayliss, Kerridge, and Verney, Earle and Cullen, and Myers and Muntwyler all show that for a particular procedure the colorimetric corrections group themselves around an average value. For statistical studies, at least for bloods from certain groups of individuals, the use of the average correction will yield a high percentage of results which will agree sufficiently closely with the hydrogen electrode values. The recent paper of Earle and Cullen confirms Cullen's correction for normal humans. The

* Both colorimetric and electrometric determinations run at the same temperature but other than 38°, usually 20°.
† Bloods studied were horse (5), dog (1), rat (1), normal human (2).

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data of Myers and Muntwyler show that certain pathological conditions yield a high percentage of bloods having the same average correction as normal bloods. The studies of Austin, Stadie, and Robinson and of Hastings demonstrate that in certain pathological conditions the correction varies from the normal so much that erroneous information would be obtained by subtracting a normal average correction value from the colorimetric readings. In view of these facts care must be taken in drawing conclusions from individual colorimetric determinations on bloods from pathological human cases until much more complete studies have been made and the variation in the colorimetric corrections more completely determined.

Dog serum has a higher colorimetric correction than human serum; that is, the difference between the colorimetric and electrometric pH values of dog serum is greater than that of normal human serum. It is not surprising therefore that variations in the correction are more frequently encountered. Undoubtedly the average Cullen correction for a large series of normal dogs would not vary far from the value of 0.35 pH originally determined by Cullen. But in the paper here reported as well as in other publications the striking variations in the colorimetric corrections which may occur even in blood from a single animal when subjected to a heavy hemorrhage emphasize the need for care in using a colorimetric method for determining variations in pH of blood in an experimental animal. Even Myers and Muntwyler report, in a series of six sera obtained from one dog, Cullen corrections varying from 0.27 to 0.43.

SUMMARY.

Comparisons were made of the hydrogen ion concentration of dog sera as determined by colorimetric methods and the hydrogen electrode method. Cullen's, Hastings' modification of Cullen's, and Dale and Evans' colorimetric methods were used. The differences between the colorimetric readings and electrometric readings (designated as colorimetric corrections) were not constant. The corrections for sera from an individual animal after severe hemorrhage showed extreme variations. The corrections are so variable that the colorimetric methods cannot be used if accurate comparisons of individual determinations are to be made.

A survey of the literature indicates that colorimetric methods may be used for statistical studies on human blood from normal and certain groups of pathological individuals with average correction values. At the present time it is doubtful if colorimetric methods should be used indiscriminately on all varieties of sera without adequate checking with the hydrogen electrode. For a comparison of individual determinations of either human or dog bloods colorimetric methods should not be used.

I wish to thank Dr. D. Wright Wilson for his interest and suggestions.

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THE MECHANISM OF EPINEPHRINE ACTION.

I. THE INFLUENCE OF EPINEPHRINE ON THE CARBOHYDRATE METABOLISM OF FASTING RATS, WITH A NOTE ON NEW FORMATION OF CARBOHYDRATES.

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Dudley and Marrian (1) were the first to show that liver glycogen disappears in fasting animals when insulin is injected. Cannon, McIver, and Bliss (2) found that insulin hypoglycemia leads to an increased discharge of epinephrine. Furthermore, there was general agreement that epinephrine mobilizes liver glycogen. By linking these three facts together, a strong case was made out for the assumption that the disappearance of liver glycogen in insulinized animals was due to the discharge of epinephrine. This assumption is contradicted by the recent observation (3) that insulin mobilizes liver glycogen in adrenalectomized animals; that is, in animals with an abolished or at least strongly reduced epinephrine secretion. A decrease in liver glycogen in adrenalectomized rats following insulin injections has been found independently by Artundo (4).

The results of Cannon and his collaborators and of other investigators are accepted as evidence that insulin evokes an increased discharge of epinephrine, while the observations on adrenalectomized animals referred to above are taken as evidence that the disappearance of liver glycogen following insulin injections is not connected with this increased release of epinephrine. In view of this it was difficult to see how the antagonistic action of insulin and epinephrine on blood sugar was brought about. The following question presented itself. If insulin alone is capable of a rapid glycogen mobilization in the liver, and if epinephrine has its chief point of attack on liver glycogen, how does epinephrine antagonize the insulin hypoglycemia? There

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was the possibility that epinephrine, whether released by the adrenals or injected, speeded up the mobilization of liver glycogen. However, there was no marked difference in the rate of disappearance of liver glycogen in normal and adrenalectomized animals, when insulin was injected. This made it unlikely that the antagonistic action between insulin and epinephrine occurred in the liver; indeed, it seemed more probable that the chief antagonistic action between these two hormones took place outside of the liver. A detailed study of the literature gave no definite clue as to the influence of epinephrine on the carbohydrate metabolism of the peripheral tissues. It appeared that the hepatic action of epinephrine had been overemphasized, with the result that an extrahepatic point of attack of this hormone was not taken into consideration. This made it desirable to supplement our knowledge of epinephrine in this respect. As in the case of

TABLE I.

Glycogen Content in Mg. per 100 Gm. of Rat.

	Liver.	Rest of body.	Total.
24 hr. fasting.....	7 ± 2	136 ± 10	143 ± 11 (Average of 16.)
48 " "	10 ± 2	111 ± 13	121 ± 13 (" " 21.)
Difference.....	+3	−25	−22

insulin, the most promising approach in the investigation of an extrahepatic action, seemed by way of a sugar balance. Experiments involving a sugar balance were carried out on sugar-fed, on rats in the postabsorptive state, and on 24 hour fasting rats. They were made in the chronological order just given, but for reasons of greater ease of presentation, the order in which these experiments are published is reversed.

Note on the New Formation of Carbohydrates.

The following observation involves the problem of new formation of carbohydrates. It was found that the glycogen content of rats undergoes only a slight diminution between the 24th and 48th hour of fasting. This is shown in the summary in Table I, which was calculated from previous experiments (5–7), including the present series of glycogen determinations in Table II. The average deviations from the mean are given in Table I in order

to illustrate the extent of individual variation. It will be noted that there is a diminution of only 22 mg. of glycogen between the 24th and the 48th hour of fasting, in spite of the fact that the animals perform muscular work during that time. It seems clear that 22 mg. of glycogen can provide only a small fraction of the lactic acid needed for the performance of muscular work, and it is therefore evident that much more than 22 mg. of glycogen is split into lactic acid during a period of 24 hours. Nevertheless, the glycogen content of the rats remains practically constant. This may have two causes. Either glycogen is not used up, because the oxidation of fat furnishes the energy for the reconversion of lactic acid into glycogen, or glycogen is formed from non-carbohydrate material as fast as it is used up. In the latter case sugar formed from non-carbohydrate sources in the liver must be transported to the muscles by means of the blood stream.

The view that fat oxidation may furnish the energy for the reconversion of lactic acid is held by Lusk (8). Krogh and Lindhard (9), in their study of muscular work, came to the conclusion that the R.Q., as determined, is always a mixture of anabolic and catabolic processes. With an R.Q. above 0.9 sugar is converted in part into fat; with an R.Q. below 0.8 the reverse process takes place. A number of investigators came to regard the conversion of fat into sugar as a proved fact, even though the Danish authors stated expressly that they were proposing only a working hypothesis. According to this hypothesis, part of the fat undergoing oxidation passes through a carbohydrate stage before it appears as CO_2 and H_2O. There can be no objection to this view, because the sugar stage in the breakdown of the fat molecule is conceived as transitory and is regarded as taking place in the same cell in which the oxidation of fat is completed. A transportation of the sugar formed from fat, from one part of the body to another, is not postulated. If such a transportation would take place, the sugar formed from fat should give rise to the excretion of sugar under special conditions. The view of Krogh and Lindhard is, therefore, not necessarily opposed to the fact that the catabolism of fat does not lead to the excretion of sugar in the diabetic organism.

The constancy of the glycogen of fasting rats is best explained either by the view of Lusk, or by the conception of Krogh and

Lindhard. Those, who believe that the energy for the reconversion of lactic acid into glycogen can come from the oxidation of carbohydrates only, will favor the latter view. The constancy of the glycogen is less satisfactorily explained by the assumption of Chaikoff and Macleod (10). These authors believe that the liver converts fat into sugar and has therefore an R.Q. of about 0.3. The sugar formed from fat in the liver is carried to the muscles where it undergoes oxidation with an R.Q. of unity. The R.Q. of the whole animal is then the algebraic sum of the R.Q.'s of the liver and muscles. Numerous measurements have shown that the R.Q. of 24 and 48 hour fasting rats is invariably in the neighborhood of 0.71. In order to explain these R.Q.'s by Macleod's theory, one has to assume that the new formation of sugar in the liver and the oxidation of this sugar in the muscles are exactly synchronized. If at any time the sugar formed from fat were to accumulate in the body, the R.Q. would fall markedly below 0.71, which has not been observed. The same consideration applies to the diabetic R.Q. The sugar formed from fat in the diabetic organism, instead of being excreted in the urine, must be carried to the muscles and must be oxidized as fast as it is formed. If any sugar formed from fat would escape in the urine, the R.Q. would fall to a low level. Lusk (11) writes that the establishment of the diabetic quotient at a level of 0.69 carries the refutation of the idea that fat may be converted into sugar. The amino acids, which are convertible into glucose in the liver of the diabetic animal, appear as sugar in the urine. It is difficult to see why the sugar formed from fat in the liver, as postulated by Macleod's theory, should not have the same fate.

It is proposed to make a distinction between sugar formation from non-carbohydrate sources as an intermediary step of oxidation, and gluconeogenesis (or new formation of carbohydrates). The former case may be called intermediary sugar formation, denoting the molecule from which the sugar is formed (fat, protein, *etc.*). The latter term should be reserved for cases where the sugar formed from non-carbohydrate sources becomes stabilized and is, therefore, capable of becoming blood sugar and of being transported to other parts of the body. The conception of Krogh and Lindhard affords an example for intermediary sugar formation from fat. An example for gluconeogenesis is sugar

formation from protein in the diabetic animal. The authors believe that it is permissible to speak of intermediary sugar formation from fat in the same way as one discusses various possibilities for intermediary stages of carbohydrate and protein oxidation. However, gluconeogenesis from fat is still awaiting experimental verification.

Experiments with Epinephrine.

The foregoing discussion may serve as a background for the changes observed in liver and body glycogen and in respiratory metabolism in 24 hour fasting rats, following the subcutaneous injection of epinephrine. Rats of 125 to 145 gm. of body weight were fasted for 21 hours. A metabolism fore period of 3 hours

TABLE II.

Glycogen Content of 24 Hour Fasting Rats.

Body weight.	Liver weight per 100 gm. of body weight.	Glycogen content per 100 gm. of body weight.			Blood sugar.
		In liver.	In other tissues.	Total.	
gm.	gm.	mg.	mg.	mg.	mg.
123	3.42	3.0	123.0	126	85
132	3.58	4.2	129.8	134	93
130	3.62	9.9	144.1	154	87
141	3.48	7.1	114.9	122	81
	3.52	6.0	128.0	134	87

was made, 0.02 mg. of epinephrine per 100 gm. of body weight was injected, and immediately a second metabolism period of 3 hours was started. The animals were killed 3 hours after the injection, and the glycogen in the liver and the rest of the body was determined in the usual manner. The amount of glycogen present in liver and body at the time of the epinephrine injection was determined on a series of control rats (Table II).

In the experiments with epinephrine in Table III the average R.Q. of the fore period was 0.715, corresponding to a non-protein R.Q. of 0.704. This R.Q. allows for no oxidation of preformed carbohydrates. The validity of this R.Q. is established by the observation recorded in Table I, that there is a disappearance of only 22 mg. of glycogen between the 24th and the 48th hour of fasting. The average

R.Q. of the epinephrine period remained unchanged as compared with the fore period, while the O_2 consumption rose in every experiment, corresponding to an average increase in calorie production of 17.3 per cent. Epinephrine produced an increase in heat production in 24 hour fasting rats at the expense of fat oxidation,

TABLE III.

Influence of Epinephrine on Carbohydrate Metabolism of 24 Hour Fasting Rats.

Average body weight 135.0 ± 7 gm. Values calculated per 100 gm. of body weight per 3 hours.

Fore period (3 hrs.).			Epinephrine period (3 hrs.).								
					Glycogen.						
O_2	R.Q.	Total calories.	O_2	R.Q.	In liver.	In rest of body.	Total.	Urine N.	Blood sugar.	Total calories.	Increase in calories over basal.
gm.			*gm.*		*mg.*	*mg.*	*mg.*	*mg.*	*mg.*		*per cent*
0.660	0.710	2.27	0.799	0.722	28.4	58.4	86.8	8.81	105	2.61	15.0
0.768	0.734	2.50	0.844	0.717	50.2	81.9	132.1	7.00	105	2.75	10.0
0.632	0.699	2.05	0.768	0.717	45.9	65.5	111.4	8.40	137	2.50	21.9
0.654	0.720	2.13	0.782	0.718	43.1	76.5	119.6	7.96	104	2.55	19.7
0.648	0.711	2.10	0.770	0.714	32.6	62.8	95.4	11.76	100	2.50	19.0
0.703	0.714	2.28	0.839	0.701	51.8	77.2	129.0	11.37	110	2.72	19.5
0.677	0.715	2.22	0.800	0.715	42.0	71.0	113.0	9.22	110	2.60	17.3

TABLE IV.

Average Glycogen Content in Mg. per 100 Gm. of Rat.

Calculated from Tables II and III.

	Liver.	Rest of body.	Total.
Controls	6 ± 2	128 ± 9	134 ± 10
3 hrs. after epinephrine	42 ± 8	71 ± 8	113 ± 15
Difference	+36	−57	−21

since an increased nitrogen elimination in the urine was not observed. This indicates that the calorigenic action of epinephrine is not necessarily connected with an increased combustion of carbohydrates.

There was a slight but unmistakable rise in blood sugar. The control rats in Table II showed an average of 87 mg. per cent,

while 3 hours after the epinephrine injection the blood sugar averaged 110 mg. per cent. The R.Q. of the fore period and of the 3 hour period during which epinephrine acted, showed no indication of carbohydrate oxidation. Nevertheless, profound changes were produced in the distribution of glycogen by the epinephrine injections. This is the more remarkable because these changes occurred in animals which were in a state of glycogen equilibrium. It will be seen from a comparison of the glycogen values in Tables II and III that epinephrine leads to a marked increase in liver glycogen and to a diminution of body glycogen in every experiment. On an average, the glycogen in the rest of the body is found to have decreased a little more than the liver glycogen has increased (− 57 against + 36 mg.). This is shown in the summary in Table IV, which was calculated from Tables II and III. The liver glycogen which had accumulated during the 3 hours of epinephrine action, was found to have disappeared again when the rats were killed 16 hours after the injection.

An increase in liver glycogen after epinephrine has been observed previously by Loeper and Crouzon (12), by Pollak (13), and by Kuriyama (14). None of these authors determined the glycogen so soon after the epinephrine injections as in the present experiments. Pollak made rabbits glycogen-free by fasting and strychnine poisoning. After daily injections of epinephrine, a considerable amount of liver glycogen (up to 4.5 per cent) was formed, in spite of the continued fasting. Kuriyama found that the livers of rabbits which were fasted and submitted to daily epinephrine injections, contained a much larger amount of glycogen than those of merely fasted animals. On an average, the muscle glycogen of the injected rabbits was lower than that of the control rabbits. The interval between the last epinephrine injection and the glycogen determination varied between 7 and 24 hours. A single injection of epinephrine into fasted rabbits also caused an increase in liver glycogen. Recently, Markowitz (15) observed that repeated epinephrine injections into fasted, strychnine-treated rabbits lead to an accumulation of liver glycogen. Since a complete carbohydrate balance was not made, this author arrived at the conclusion that the newly formed glycogen in the liver was derived from fat.

The present experiments reveal that muscle glycogen is another

possible source for the newly formed liver glycogen. It is now definitely known, especially from the work of Mann and Magath (16) on hepatectomized dogs, that muscle glycogen is not a direct source of blood sugar after epinephrine injections, or under any other conditions. When muscle glycogen is split, the presence of the glycolytic ferment causes an immediate change of the split products into lactic acid (Lohmann (17)). The course of events in 24 hour fasting rats would then be that epinephrine mobilizes muscle glycogen and that lactic acid enters the blood stream and is carried to the liver where it is deposited as glycogen. If the disappearing muscle glycogen (57 mg.) were oxidized, the R.Q. should be 0.74, while the R.Q. actually observed was 0.715.

Before considering this explanation further, it was necessary to determine whether lactic acid was able to form liver glycogen. *d*-Lactic acid in the free state or as sodium salt, as well as *r*-sodium lactate, when fed by stomach tube to 24 hour fasting rats, gave rise to the formation of considerable amounts of liver glycogen (18). Sodium lactate, when injected subcutaneously, was far less effective in forming liver glycogen. A detailed report of these experiments will be presented in a later paper. Abramson, Eggleton, and Eggleton (19) reported recently that *r*-sodium lactate, when injected intravenously, does not form liver glycogen. It would seem that their experimental procedure was not favorable for the detection of glycogen formation from lactic acid.

With the demonstration that lactic acid forms liver glycogen, one possible objection to the explanation here proposed has been eliminated. An older observation gains special significance in the light of the present work. It was found that epinephrine injections lead to a marked increase in the blood lactic acid of rabbits and cats (20). Tolstoi, Loebel, Levine, and Richardson (21) observed an increase in blood lactic acid in men after epinephrine injections. An increase was also found, to a lesser extent, in the rats used for the present experiments.

There is the possibility that the liver glycogen accumulating during epinephrine action is derived from non-carbohydrate sources, in other words, that one is dealing with gluconeogenesis according to the definition given in the preceding section. If this were the case, one would have to assume that the glycogen disappearing from the rest of the body is oxidized. The simultaneous

occurrence of the two processes (anabolic and catabolic) would also result in the R.Q. actually observed. The average nitrogen elimination in the 3 hour epinephrine period was 9.2 mg. If one assumes a conversion into glucose corresponding to a D:N ratio of 3.65, 34 mg. of glucose could have been derived from the catabolism of protein. The increase in liver glycogen of 36 mg. is of the same magnitude. This indicates that it is unnecessary to assume gluconeogenesis from fat in order to explain the increase in liver glycogen after epinephrine injections. We believe that gluconeogenesis does not explain the results, because an increase in liver glycogen was also observed in rats in the postabsorptive state in which gluconeogenesis should be in abeyance on account of previous carbohydrate feeding and an R.Q. above 0.8. The increase in blood lactic acid is also in favor of the first explanation.

The experiments on 24 hour fasting rats recorded in this paper show in a definite way that epinephrine influences the carbohydrate metabolism of the peripheral tissues, since it leads to the disappearance of muscle glycogen. The experimental demonstration of glycogen formation in the liver from lactic acid supports the conclusion that glycogen mobilized in the muscles is converted into liver glycogen with lactic acid as an intermediary stage. Muscle glycogen is therefore an indirect source of blood sugar if the liver is present and if there is an escape of lactic acid from the muscles. Epinephrine injections, violent exercise, strychnine and insulin convulsions, asphyxia, decerebration, and a variety of other conditions lead to an escape of lactic acid from the muscle. The loss of muscle glycogen incurred under these different conditions is repaid, in part at least, by the liver in terms of glucose. It remains an established fact that the liver is the only direct source of blood sugar in the body. In the absence of the liver, muscle glycogen is unable to contribute sugar to the blood, because lactic acid cannot be converted into liver glycogen.

SUMMARY AND CONCLUSIONS.

1. Between the 24th and 48th hour of fasting, the glycogen content of rats diminishes by only 22 mg. This constancy of the glycogen during fasting is discussed from the standpoint of the new formation of carbohydrates. It is proposed to make a

sharp distinction between carbohydrate formation from protein or fat as an intermediary step in the complete oxidation of these molecules and gluconeogenesis. The latter term should be reserved for cases where the sugar formed from non-carbohydrate sources becomes stabilized.

2. The R.Q. of 24 hour fasting rats of 0.715 remained unchanged when epinephrine was injected. The O_2 consumption rose in each experiment, corresponding to an average increase in heat production of 17.3 per cent.

3. 3 hours after the subcutaneous injection of epinephrine (0.02 mg. per 100 gm. of rat), the body glycogen (mainly muscle glycogen) diminished on an average by 57 mg., while the liver glycogen increased by 36 mg.

4. The increase in liver glycogen after epinephrine injections is explained by the conversion of muscle glycogen into liver glycogen with lactic acid as an intermediary stage. This explanation is supported by the fact that the oral administration of lactic acid has been found to lead to the deposition of liver glycogen. Another possibility which has been considered is the conversion of protein into liver glycogen, with a simultaneous oxidation of the glycogen disappearing in the muscles. The combination of the two processes (anabolic and catabolic) would also result in the R.Q. actually observed.

5. Accepting the first explanation as the better supported one, it follows that muscle glycogen is an indirect source of blood sugar, if the liver is present and if there is an escape of lactic acid from the muscles.

6. Epinephrine influences the carbohydrate metabolism of the peripheral tissues, since it leads to the disappearance of muscle glycogen.

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THE MECHANISM OF EPINEPHRINE ACTION.

II. THE INFLUENCE OF EPINEPHRINE AND INSULIN ON THE CARBOHYDRATE METABOLISM OF RATS IN THE POSTABSORPTIVE STATE.*

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The experiments on 24 hour fasting rats recorded in the preceding paper (1) have shown that epinephrine injections lead to a disappearance of muscle glycogen and to an accumulation of liver glycogen. The glycogen mobilized in the muscles was apparently not oxidized, and it was suggested that muscle glycogen was converted into liver glycogen with lactic acid as an intermediary stage. Before describing the present experiments it may be advisable to examine the evidence so far accumulated as to the behavior of liver and muscle glycogen in animals in the postabsorptive state after epinephrine injections.

HISTORICAL.

Shortly after Blum (2) had discovered in 1901 that injections of adrenalin cause glycosuria, several authors attempted to investigate the influence of such injections on the glycogen stores of the body. In some of these investigations, methods were used which were shown later to account for only fractions of the glycogen present (Doyon and Kareff (3), Doyon, Morel, and Kareff (4)). These will not be considered here in detail. In 1905 Bierry and Gatin-Gruzewska (5), using Pflüger's method, determined the liver glycogen of four dogs, 4 to 5 hours after an adrenalin injection. The values found varied from 0.16 to 1.5 per cent. Control values are not recorded, which is a serious objection in view of the results obtained in another paper of Gatin-Gruzewska (6). Here adrenalin was given to 24 hour fasting rabbits and the animals were killed by bleeding 32 to 40 hours after the injection. Neither muscle nor liver glycogen was found, but the control animals also contained no liver glycogen and only 0.05 per cent

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muscle glycogen. Paton (7) and Drummond and Paton (8) concluded from experiments in which liver glycogen was determined in three injected and two control rabbits that it decreases after the injection of one large dose but not when adrenalin is given repeatedly for several days. Agadschanianz (9) working with the old water extraction method and with Pflüger's method but weighing the glycogen (both not quantitative procedures), killed rabbits 36 hours after the injection of 1.0 to 1.5 mg. of adrenalin. The values for liver glycogen of his injected rabbits were 0, 0, 0.09, 0.11, for the controls 0.06, 0.6, 0,0.27 per cent. No muscle glycogen was found in the injected and only 0.05, trace, 0.025, and 0.1 per cent in the control rabbits. Doyon and Gautier (10) took liver samples from the same dog before and 26 to 45 minutes after the injection of large doses of epinephrine into a mesenteric vein. In all cases there was a decrease in liver glycogen. (8.05 to 3.66, 8.04 to 5.05, 7.9 to 5.7, 5.48 to 3.77, 5.38 to 3.74). Control experiments without injection were not performed.

This is the principal evidence to be found in the literature on a decrease in liver and muscle glycogen after epinephrine injections. The objections to these papers are obvious. In view of the great variability of liver and muscle glycogen of the species used, a sufficient number of animals has not been used by any of the authors. The extremely low glycogen values of most of the controls cast doubt on the analytical technique of several workers. Postmortem glycogenolysis was not sufficiently recognized at that time and probably no precautions were taken to avoid it. Bleeding the animals to death is also an unsuitable procedure because it leads to premortal glycogenolysis. Where liver glycogen was determined on the same animal before and after the injection, narcosis and laparotomy had to be resorted to, procedures which in themselves lower the liver glycogen. In many instances the animals were killed 1 or even 2 days after the injection at a time when epinephrine had long ceased to act. In spite of these shortcomings this work has been accepted as evidence that epinephrine causes a decrease in liver glycogen and the authors mentioned are usually quoted together in text-books and reviews as having proved this point (*cf.* the extensive review by Trendelenburg (11)). When it was found that muscle glycogen is not a direct source of blood sugar[1] (Velich (13), Vandeput (14), Falta and Priestley (15)), the claim of the above authors that epinephrine leads to a decrease in muscle glycogen was disregarded. In this way the hepatic action of epinephrine was given a predominant rôle, while an extrahepatic point of attack was gradually left out of consideration. The high blood sugar was taken as further evidence that the liver glycogen diminishes after epinephrine, since it seemed inconceivable that a hyperglycemia could develop without a decrease in liver glycogen.

Another group of authors (Loeper and Crouzon (16), Pollak (17), Kuriyama (18)) found that liver glycogen increases in fasting animals after epinephrine injections, in spite of a marked hyperglycemia and usually also

[1] It is only since the experiments of Mann (12) that this may be regarded as a well established fact.

glycosuria. A larger number of animals was used in the work of the last two authors, and there was little variability in the controls on account of the longer fasting period. Since no satisfactory explanation could be offered for this paradoxical behavior of liver glycogen, the results of Pollak and Kuriyama did not lead to a reconsideration of the mechanism of epinephrine action. Recently Junkersdorf and Schüler (19) and Junkersdorf and Török (20) found higher liver glycogen in well fed dogs receiving epinephrine than in suitable controls. In underfed dogs rather the opposite tended to be the case. Under both conditions the muscle glycogen of the injected dogs was markedly lower. Markowitz (21) also observed an increase in liver glycogen in fasting rabbits receiving daily epinephrine injections.

In summarizing one may say that it has not been proved definitely that liver glycogen diminishes after epinephrine injections, nor are there any data available which indicate how much liver glycogen disappears. Future work will probably show that the liver glycogen diminishes under certain conditions, especially if there is strong glycosuria, but this is still awaiting experimental verification. On the other hand, liver glycogen has been found to increase after epinephrine injections. A decrease in muscle glycogen has been claimed by all workers who analyzed for it, though much of the work has not been sufficiently controlled.

It was rather surprising to find, by going back to the sources, that these contradictory results form the basis for the present explanation of epinephrine hyperglycemia and glycosuria. According to this explanation hyperglycemia and glycosuria develop because a larger amount of sugar is given off by the liver than can be cared for by the tissues. It has apparently not been considered that there is, as a rule, not enough liver glycogen present to supply the body for hours with amounts of sugar greater than the normal tolerance limit of the tissues. The fact that liver glycogen is sometimes higher after epinephrine injections should have cast doubt on the assumption that the only cause of the hyperglycemia consists in a mobilization of liver glycogen. By assigning a purpose to the epinephrine hyperglycemia, namely, to facilitate utilization of blood sugar in the muscles, attention has been distracted further from the cause of the hyperglycemia. In the present paper a new conception of the mechanism of epinephrine action is advanced and it is shown that there are other factors involved in the production of hyperglycemia besides mobilization of liver glycogen.

In the present experiments the animals were rich in glycogen, while the experiments on 24 hour fasting rats (1) were made on animals with low carbohydrate reserves. There is also this differ-

ence between the experiments on 24 hour fasting rats and rats in the postabsorptive state. In the former animals the glycogen is in a stationary condition at the time the epinephrine injections are made; in the latter animals glycogen disappears even if no epinephrine is injected. As will be shown later, epinephrine greatly accelerates the disappearance of muscle glycogen in rats in the postabsorptive state; at the same time it leads to an accumulation of liver glycogen. The changes in glycogen are therefore of the same type as in 24 hour fasting rats.

A method has been worked out which permits the calculation of the amounts of glycogen disappearing from liver and muscles of rats in the postabsorptive state. This method is described in detail below. The simultaneous determination of sugar oxidation makes it possible to strike a sugar balance, because one is in a position to judge how much of the disappearing glycogen is accounted for by sugar oxidation.

The following three series of experiments have been made on rats in the postabsorptive state: one series on control rats which received an injection of saline; a second series with the subcutaneous injection of 0.02 mg. of epinephrine per 100 gm. of rat which was also the dose employed in the experiments on 24 hour fasting rats; finally, a third series with the injection of 0.75 units of insulin per 100 gm. of rat. In each case the animals were killed 3 hours after the injection.

Method of Determining the Disappearance of Glycogen in Rats in the Postabsorptive State.

Male rats of 130 to 160 gm. of body weight were fasted for 24 hours. The urine of the last 17 hours was collected for nitrogen determinations. 2.5 cc. of a glucose solution, containing 1.065 gm. of glucose per 100 gm. of body weight, were fed by stomach tube. This is the average amount of glucose absorbed in 4 hours by 24 hour fasting rats (22). The respiratory metabolism was determined during the 4 hours of glucose absorption. Since the amount of glucose absorbed and oxidized during these 4 hours is known, one can calculate how much glycogen is being deposited during that time. (This calculation is made on the basis of previous experiments (22) in which the average amount of absorbed glucose accounted for by sugar oxidation and glycogen formation

TABLE I.

Influence of Epinephrine and Insulin on Sugar Oxidation and Glycogen Content of Rats in the Postabsorptive State.

Values calculated per 100 gm. of body weight.

Glucose absorption period (4 hrs.).				Postabsorptive period (3 hrs.)*							
							Glycogen.				
O_2	R.Q.	Glucose absorbed.	Glucose oxidized.	O_2	R.Q.	Glucose oxidized.	In liver.	In rest of body.	Total.	Urine N per hr.	Blood sugar.
				Controls, saline injection.							
gm.		*gm.*	*gm.*	*gm.*		*gm.*	*gm.*	*gm.*	*gm.*	*mg.*	*mg.*
0.795	0.857	1.087	0.356	0.567	0.859	0.255	0.210	0.345	0.555	2.85	127
0.899	0.844	1.042	0.367	0.628	0.801	0.169	0.154	0.254	0.408	2.83	99
0.842	0.887	1.067	0.458	0.635	0.847	0.263	0.164	0.243	0.407	2.95	102
0.903	0.885	1.064	0.488	0.659	0.809	0.195	0.157	0.215	0.372	2.82	123
0.860	0.868	1.065	0.417	0.622	0.829	0.220	0.171	0.264	0.435	2.86	113
				Epinephrine injection.							
0.836	0.832	1.034	0.304	0.765	0.844	0.312	0.226	0.139	0.365	3.25	176
0.846	0.859	1.066	0.383	0.749	0.821	0.254	0.269	0.139	0.408	2.88	191
0.913	0.870	1.080	0.448	0.736	0.825	0.255	0.226	0.135	0.361	3.11	148
0.893	0.879	1.077	0.463	0.712	0.831	0.261	0.257	0.124	0.381	2.87	158
0.961	0.875	1.022	0.482	0.771	0.811	0.231	0.239	0.113	0.352	3.64	196†
0.889	0.863	1.056	0.416	0.747	0.826	0.263	0.244	0.130	0.374	3.15	174
				Insulin injection.							
0.782	0.838	1.069	0.293	0.589	0.932	0.400	0.129	0.334	0.463	3.30	53
0.838	0.860	1.022	0.380	0.613	0.946	0.448	0.050	0.249	0.299	2.73	72
0.936	0.862	1.076	0.438	0.616	0.945	0.450	0.075	0.194	0.269	2.76	69
0.794	0.902	1.046	0.468	0.586	0.951	0.437	0.084	0.223	0.307	2.87	81
0.837	0.865	1.053	0.395	0.601	0.943	0.434	0.085	0.250	0.335	2.92	69

* Injections were made at the beginning of the postabsorptive period.

† Double dose of epinephrine (0.04 mg. per 100 gm.) injected. 10 mg. of sugar excreted in urine.

in liver and the rest of the body was 86.5 per cent.) 4 hours after the glucose feeding the animals were in the postabsorptive state. Then the injections of saline, epinephrine, or insulin were made. The respiratory metabolism was determined for 3 hours after the injection. After this time the animals were killed and the glycogen content of the liver and the rest of the body was determined in the usual manner. Since one knows how much glycogen is present at the time of injection as well as 3 hours after the injection, the former by computation and the latter by direct determination, one can calculate how much glycogen disappears during 3 postabsorption hours, and one can compare this with the amount of glucose that has been oxidized during that time.

The following example (Rat 4 of the control series in Table I) illustrates how this calculation is carried out.

4 Hour Glucose Absorption Period.

Weight of rat 132.5 gm. Glucose fed by stomach tube 1.41 gm. Per 100 gm. of body weight per 4 hours: glucose absorbed 1.064 gm.; glucose oxidized 0.488 gm. In Table XI of a previous paper (22) an average of 86.5 per cent of the absorbed glucose is accounted for. The quotient $\frac{\text{glycogen formed in liver}}{\text{total glycogen formed}}$ is 0.422 and the quotient $\frac{\text{glycogen formed in the rest of the body}}{\text{total glycogen formed}}$ is 0.577.

On this basis one finds:

$\frac{1.064 \times 86.5}{100} = 0.920$ gm. of absorbed glucose accounted for.

$0.920 - 0.488 = 0.432$ gm. of glucose converted into glycogen.

$0.432 \times 0.422 = 0.182$ gm. of glucose converted into liver glycogen.

$0.432 \times 0.577 = 0.250$ gm. of glucose converted into body glycogen.

In order to find the total amount of glycogen present at the time of injection, add preformed glycogen of 24 hour fasting rats (22) (8 mg. for liver and 140 mg. for the rest of the body).

$0.182 + 0.008 = 0.190$ *gm. of glycogen present in the liver.*

$0.250 + 0.140 = 0.390$ *gm. of glycogen present in the rest of the body.*

3 Hour Period after the Injection. (Postabsorptive Period).

The glycogen determinations 3 hours after the injection, calculated per 100 gm. of body weight, gave the following values: liver 0.157, body 0.215 gm.

Hence, $0.190 - 0.157 = 0.033$ *gm. of glycogen disappeared from the liver.*

$0.390 - 0.215 = 0.175$ *gm. of glycogen disappeared from the rest of the body.*

Owing to the difference in blood sugar concentration at the beginning (158 mg. per cent) and at the end of the 3 hour period (123 mg. per cent), there is also a disappearance of glucose from the blood and body fluids. It is assumed that the blood is in equilibrium with 50 per cent of the body weight. Therefore: $\frac{158}{2} - \frac{123}{2}$ = *18 mg. of glucose disappeared from the blood and body fluids.*

The total amount of glucose which disappeared during the 3 hours after the injection was 0.033 + 0.175 + 0.018 = 0.226 gm. The glucose oxidized

TABLE II.

Glycogen Content of Liver and Body at Time of Injections.

Calculated from Table I.

	Liver.	Rest of body.	Total.
	mg.	*mg.*	*mg.*
Controls, saline injection.	254	478	732
	233	449	682
	204	409	613
	190	390	580
	220	432	652
Epinephrine injection.	257	481	738
	235	452	687
	213	421	634
	206	411	617
	178	372	550
	218	427	645
Insulin injection.	275	505	780
	221	431	652
	216	425	641
	192	393	585
	226	438	664

during that time, as calculated from the non-protein R.Q., was *0.195 gm.* Hence, 86.4 per cent of the calculated loss of glucose has been accounted for by oxidation.

Results.

Table I contains the data from which the calculations in the manner just described have been made. The amounts of glycogen present in liver and body at the time of the injections, as calcu-

lated from Table I, are shown in Table II. It will be noted in Table II that saline, epinephrine, and insulin were injected into animals containing very similar quantities of glycogen. The average glycogen content of the liver in the three cases was 220, 218, and 226 mg., and of the rest of the body 432, 427, and 438 mg. The glycogen values observed 3 hours after the injections are recorded in Table I. By subtracting the glycogen values in Table I from those in Table II one obtains the values recorded in Table III.

TABLE III.

Disappearance of Glycogen (in Mg. of Glucose) from Liver and Rest of Body during 3 Postabsorption Hours.

Calculated from Tables I and II.

	Liver glycogen.	Body glycogen.	Loss of sugar from body fluids.	Total carbohydrate disappeared.	Carbohydrate oxidised.
Controls, saline injection.	−44	−133	−15	192	255
	−79	−195	−30	304	169
	−40	−166	−23	229	263
	−33	−175	−18	226	195
Epinephrine injection.	−31	−342	+ 9	364	312
	+34	−313	+17	262	254
	+13	−286	− 5	278	255
	+51	−287	± 0	236	261
	+61	−259	+19	179	231
Insulin injection.	−146	−171	−52	369	400
	−170	−182	−43	395	448
	−141	−231	−45	417	450
	−108	−170	−44	322	437

First a comparison will be made between the carbohydrate oxidation of the glucose absorption and the postabsorptive period. The control animals in Table I oxidized an average of 417 mg. of sugar in the 4 hour glucose absorption period. In the 3 hour postabsorptive period which follows they oxidized 220 mg. of sugar. In order to compare these two values they are calculated per hour; namely, 104 mg. oxidized in the absorption period, and 73 mg. oxidized in the postabsorptive period. This indicates that as soon as the supply of glucose from the intestine subsides, there

is a decrease in carbohydrate oxidation. The same is true for the animals which received epinephrine after the glucose absorption period. In the absorption period they oxidized 104 mg. per hour, against 87 mg. per hour in the postabsorptive period. In contrast to this, the animals which received insulin after the glucose absorption period showed an increase in carbohydrate oxidation. In the absorption period they oxidized 99 mg. per hour; in the postabsorptive period the oxidation rose to 145 mg. per hour.

Secondly, a comparison will be made between the amounts of glycogen disappearing from liver and body during 3 postabsorption hours following saline, epinephrine, and insulin injections. The individual experiments are recorded in Table III. It will be

TABLE IV.

Disappearance of Glycogen (in Mg. of Glucose) from Liver and Rest of Body during 3 Postabsorption Hours.

Average values of Table III.

	Liver glycogen.	Body glycogen.	Loss of sugar from body fluids.	Total carbohydrate disappeared.	Carbohydrate oxidized.	Blood sugar.
Controls, saline injection.....	−49	−167	−22	238	220	113
Epinephrine injection.....	+26	−298	+8	264	263	174
Insulin injection.....	−141	−188	−47	376	434	69

sufficient to consider the average values of Table III which are given in Table IV. The control rats in Table IV utilized 49 mg. of liver and 167 mg. of body glycogen (mainly muscle glycogen). The animals receiving epinephrine utilized apparently no liver glycogen, since there was an increase in liver glycogen of 26 mg. All the glycogen which the animals receiving epinephrine utilized came from the rest of the body. The important point is that nearly twice as much body glycogen disappeared after epinephrine injections than under normal conditions or after insulin injections. The insulinized rats utilized 3 times more liver glycogen but only slightly more body glycogen than the control rats. Contrary to expectation it is not epinephrine but insulin which causes a marked disappearance of liver glycogen in rats in the postabsorptive state.

The insulinized rats utilize the largest amount of liver glycogen, the rats receiving epinephrine the largest amount of body glycogen, while the normal rats hold an intermediate position. The calculated disappearance of carbohydrates shows a fair agreement with the observed oxidation of carbohydrates. In the normal animals in Table IV 7.5 per cent more carbohydrate disappears than is accounted for by oxidation; in the animals receiving epinephrine the agreement happens to be perfect, which is undoubtedly a coincidence, while in the insulinized animals 13.4 per cent more sugar is oxidized than is accounted for by the calculated disappearance of sugar. If insulin would cause the conversion of part of the disappearing sugar into an unknown intermediary substance, more sugar should be lost sight of than is accounted for by oxidation. Here the opposite condition obtains, definitely disproving this hypothesis.

Corresponding to the fall in blood sugar, glucose disappeared from the blood and body fluids of the control and insulinized rats, while glucose accumulated in the rats receiving epinephrine because hyperglycemia developed (Table IV). At the end of the 3 hour postabsorptive period the average blood sugar of the normal rats was 113, of the rats receiving epinephrine 174, and of the insulinized rats 69 mg. per cent (Table I). The dose of epinephrine administered produced no glycosuria. In Rat 5, marked with a dagger in Table I, the double dose of epinephrine (0.04 mg. per 100 gm. of rat) was given, with the result that 10 mg. of sugar were excreted in the urine. It was not intended to produce glycosuria in these experiments; the aim was to keep the dose as small as possible and yet to obtain a clear effect on carbohydrate metabolism. In this way it was found that a dose that would be pronounced small, when judged by its effect on blood sugar, has a very marked effect on the disappearance of muscle glycogen. It is therefore not necessary to inject a large dose in order to demonstrate an extrahepatic action of epinephrine. How far the dose of epinephrine here employed may be regarded as physiological is discussed in the following paper (23).

Influence of Epinephrine and Insulin on Heat Production.

The data on respiratory metabolism in Table I are expressed in terms of calories in Table V. First a comparison will be made

between the average calorie production of the glucose absorption and the postabsorptive period. The control and insulinized animals showed a slightly lower heat production in the postabsorptive period as compared with the absorption period. In the former animals the total calories diminished by 3.6, and in the latter animals by 3.8 per cent. In contrast to this, the heat production rose when epinephrine was injected; namely, from 0.76 calories per hour in the absorption period to 0.86 calories per hour in the postabsorptive period, corresponding to an increase of 12.2 per cent. Next the average calorie production of the normal rats in the postabsorptive state is compared with that of the rats in the

TABLE V.

Influence of Epinephrine and Insulin on Heat Production of Rats in the Postabsorptive State.

Values per 100 gm. of body weight. Calculated from Table I.

	Glucose absorption period (4 hrs.).					Postabsorptive period (3 hrs.).				
	Calories from:			Total calories.	Total calories per hr.	Calories from:			Total calories.	Total calories per hr.
	Protein.	Fat.	Carbohydrate.			Protein.	Fat.	Carbohydrate.		
Control	0.29	1.02	1.56	2.87	0.72	0.21	1.03	0.83	2.07	0.69
Epinephrine*	0.31	1.19	1.56	3.06	0.76	0.24	1.35	0.98	2.57	0.86
Insulin*	0.29	1.09	1.48	2.86	0.71	0.22	0.22	1.62	2.06	0.69

*Injected at the beginning of the postabsorptive period.

postabsorptive state receiving insulin and epinephrine injections. The calories of the control and insulinized animals were within 0.5 per cent the same, in spite of the fact that the latter animals oxidized 97.3 per cent more carbohydrates than the former. The insulinized animals produced 0.79 more carbohydrate and 0.81 less fat calories than the control animals, in other words, there occurred an equicaloric replacement of carbohydrate oxidation for fat oxidation after the insulin injection. This perfect replacement is quite remarkable and it explains why insulin does not have

a calorigenic action.[2] On the other hand, when epinephrine was injected, the heat production was 24 per cent higher than in the normal animals in the postabsorptive state.[3] The animals receiving epinephrine produced 0.32 more fat and 0.15 more carbohydrate calories than the control animals. Therefore, 67 per cent of the extra calories was furnished by fat and 33 per cent by carbohydrates. Since in the control animals only 55 per cent of the non-protein calories was derived from fat, epinephrine increased fat oxidation proportionately more than carbohydrate oxidation. If no carbohydrates are being oxidized at the time of the epinephrine injections, as in the experiments on 24 hour fasting rats (1), the extra heat is furnished exclusively by fat oxidation. The mechanism by which epinephrine increases fat oxidation is still uncertain. There may be a direct accelerating influence on fat oxidation, or fat metabolism may be influenced indirectly through an as yet unknown mechanism.

Utilization of Liver and Body Glycogen in Normal Rats in the Postabsorptive State.

The utilization of liver and body glycogen in normal rats in the postabsorptive state is of interest from the standpoint of the physiology of fasting. The control rats in Table IV utilized 216 mg. of glycogen, 23 per cent being derived from the liver and 77 per cent from the rest of the body. Body glycogen (chiefly muscle glycogen) is therefore utilized to a larger extent in the first 3 postabsorption hours than liver glycogen. When the fast has lasted for 24 hours, neither liver nor body glycogen disappears. The glycogen content of the rats remains practically constant, there being a diminution of only 22 mg. of glycogen between the 24th and 48th hour of fasting (1). It is of interest to inquire how soon this stationary condition of the glycogen is reached and what metabolic adjustments have to take place until the glycogen can remain constant. If the utilization of liver and body glycogen were going on at the same rate in later periods of fasting as in the first 3 post-

[2] Hawley and Murlin (24) observed in rabbits increased heat production from fat in the 1st hour of insulin action but not in later periods. This would escape attention in our experiments which extend over 3 hours.

[3] Boothby and Sandiford (25) using dogs, observed an increased heat production of a similar magnitude after physiological doses of epinephrine.

absorption hours, the 24 hour fasting level would be reached by the liver in 13 and by the body in 5 hours. There is reason to believe that the rate of glycogen utilization diminishes as fasting proceeds, because the insulin production of the pancreas will adjust itself to the fasting condition. Utilization of liver glycogen is greatly increased when insulin is present in excess during fasting. It will be noted in Table IV that the insulinized rats utilized 3 times more liver glycogen than the normal rats in the postabsorptive state. A decrease in the insulin production of the pancreas which is known to occur during fasting[4] will therefore make for a longer preservation of liver glycogen. At or before 24 hours of fasting most of the liver glycogen is used up and the body glycogen has attained a stationary condition. If the body glycogen is to reach this stationary state, carbohydrate oxidation has to be replaced by fat oxidation. Presumably, this metabolic replacement cannot be carried to completion until the insulin production of the pancreas has been reduced to a minimum. Since insulin and epinephrine act antagonistically, a low supply of the former may mean a relative preponderance of the latter. It is possible that the preservation of a normal blood sugar level during fasting is in some way connected with the epinephrine discharge of the adrenals. This is suggested by the observation that adrenalectomized rats developed hypoglycemia after a fasting period of 24 hours and that the livers of such animals contained only traces of glycogen (26). A similar observation was made on adrenalectomized mice after shorter periods of fasting and in three cases typical hypoglycemic convulsions developed after 5 hours of fasting. The cycle of carbohydrates which is observed after small doses of epinephrine (see the following discussion) may play a rôle in the preservation of liver glycogen and hence of a normal blood sugar level during fasting. This cycle proceeds in the following manner. Lactic acid derived from muscle glycogen is converted into liver glycogen and glucose derived from liver glycogen is returned to the muscles. In the absence of carbohydrate oxidation this cycle can go on without any loss of carbohydrate and may therefore explain why glycogen is always found in the liver of fasting animals, making unnecessary the assumption that gluconeogenesis occurs.

[4]Staub, H., *Z. klin. Med.*, 1926, civ, 587.

Influence of Insulin and Epinephrine on Utilization of Liver and Body Glycogen.

The mere determination of the respiratory metabolism after insulin or epinephrine injections does not permit of a conclusive analysis of the changes in carbohydrate metabolism produced by these hormones. In the experiments in Table IV insulin increased carbohydrate oxidation by 97.3 and epinephrine by 19.5 per cent. The fact that insulin and epinephrine have an accelerating effect on carbohydrate oxidation but an opposite effect on the blood sugar level would be difficult to explain without knowledge of the underlying changes in liver and body glycogen. The increased carbohydrate oxidation after non-convulsive doses of insulin does not occur to any appreciable extent at the expense of muscle glycogen but almost exclusively at the expense of liver glycogen. It will be noted in Table IV that the animals receiving insulin oxidized only 21 mg. more of body glycogen than the normal rats in the postabsorptive state, while 3 times more liver glycogen disappeared. The rapid disappearance of liver glycogen after insulin injections is regarded as an indirect effect. It is brought about by an increased utilization of blood sugar in the peripheral tissues with a compensatory mobilization of liver glycogen. This action of insulin on rats in the postabsorptive state is analogous to the one observed on sugar-fed rats (22), where the muscles appropriated most of the absorbed sugar at the expense of glycogen deposition in the liver. According to this analysis, insulin is a hormone that leads to a preferential utilization of blood sugar and indirectly of liver glycogen, the latter being the only important direct source of blood sugar in the body.

After epinephrine injections, carbohydrate oxidation is carried on mainly at the expense of muscle glycogen. In the experiments on rats in the postabsorptive state in Table IV, the disappearance of muscle glycogen was greatly accelerated, while liver glycogen was apparently not drawn upon since it did not diminish. Utilization of blood sugar (which is derived from liver glycogen) and of muscle glycogen are therefore influenced in an opposite direction by epinephrine injections. The former is diminished, while the latter is markedly increased in rats in the postabsorptive state. This explains the otherwise unintelligible fact that epinephrine

may cause hyperglycemia and increased carbohydrate oxidation at the same time.

An increase in carbohydrate oxidation after epinephrine is observed only in rats in the postabsorptive state but not in sugar-fed or in fasting rats. The influence of epinephrine on sugar-fed animals will be discussed in a later paper (23). In 24 hour fasting rats in which carbohydrate oxidation is reduced to a minimum or absent, epinephrine injections do not lead to carbohydrate oxidation though they cause a disappearance of muscle glycogen. This indicates, together with the results obtained on sugar-fed animals, that epinephrine has not a direct accelerating influence on carbohydrate oxidation. It merely mobilizes muscle glycogen, and it depends on the metabolic state of the animal (presumably on the insulin content of the tissues) to what extent the glycogen mobilized in the muscles is being oxidized.

Carbohydrate Balance after Epinephrine.

The glycogen content of the liver observed 3 hours after the epinephrine injection may be represented as the algebraic sum of the following processes.

1. Mobilization of liver glycogen.
2. Reconversion of blood sugar into liver glycogen.
3. Conversion of blood lactic acid (from muscle glycogen) into liver glycogen.

Since glycogen disappears from the liver of the normal rat in the postabsorptive state and since there is no indication that epinephrine inhibits glycogenolysis in the liver, Process 1 must occur though the sugar balance in Table IV offers no evidence thereof. Epinephrine does not prevent Process 2, since it has no inhibiting effect on glycogen synthesis in the liver during glucose absorption (23). Process 3 has already been discussed in the preceding paper (1). Its occurrence in the rat in the postabsorptive state following epinephrine injections is indicated by the fact that more glycogen disappears from the muscles than can be accounted for by carbohydrate oxidation (Table IV). Under three different experimental conditions (24 hour fasting, rats in the postabsorptive state, and sugar-fed rats) the algebraic sum of these three processes was positive, that is, epinephrine caused an increase and not a decrease in liver glycogen. Here only the rats in the postabsorptive state

will be considered. We can leave Process 2 out of consideration for the present, because it is a function of Process 1, there being no other direct source of blood sugar than liver glycogen. The question arises as to what rate should be assigned to Processes 1 and 3. The sugar balance merely gives information as to the minimum rate of Processes 1 and 3. This is shown in the following calculation. In the experiments in Table IV the liver glycogen increased by 26 mg. At the same time 298 mg. of glycogen disappeared from the body, while only 263 mg. of sugar were oxidized. This leaves 35 mg. of glycogen for Process 3. In order to raise the blood sugar to 174 mg. per cent, a mobilization of 8 mg. of liver glycogen is required. Deducting 8 mg. from 35 mg. one obtains the end result of plus 27 mg. of liver glycogen, 26 mg. being the figure actually observed. The conditions of the sugar balance are fulfilled but no room is left for mobilization of liver glycogen and hence for utilization of blood sugar in the tissues. If one allows for blood sugar utilization, more liver glycogen must be mobilized to supply sugar, and consequently, since the liver glycogen is not found to diminish, a greater rate of Process 3 is required. In this way a cycle of carbohydrates is established. Blood sugar derived from liver glycogen is utilized in the muscles and at the same time lactic acid derived from muscle glycogen is transported back to the liver to replenish the glycogen which has been lost.[5]

It is evident that the rate of this cycle is determined by the rate of blood sugar utilization. In the normal and insulinized animals, in which Process 3 is not occurring, the amount of liver glycogen that disappears can be used as an index of blood sugar utilization. There was a disappearance of 49 mg. in the control and of 141 mg. in the insulinized animals (Table IV). To these must be added 22 mg. of sugar derived from the body fluids in the former and 47 mg. in the latter animals, giving a total blood sugar utilization of 71 and 188 mg. respectively. As has already been pointed out, epinephrine certainly does not inhibit glycogen mobilization in the liver. If therefore as much liver glycogen is mobilized as in

[5] This cycle is a necessary supposition, if utilization of blood sugar occurs after epinephrine injections, unless one makes the assumption that liver glycogen is formed from non-carbohydrate sources as fast as it disappears. As has been set forth in the preceding paper (1) there is no convincing evidence for the second alternative.

the normal rats in the postabsorptive state, 49 mg. of sugar may be utilized in the peripheral tissues of the rats receiving epinephrine injections. This requires a return of 73 mg. of lactic acid from the muscles in order to account for the end result of plus 26 mg. of liver glycogen. The mobilization of liver glycogen will be greater than 49 mg. in case blood sugar is reconverted into liver glycogen (Process 2). Process 2 can only occur to any larger extent in animals in the postabsorptive state following epinephrine injections, if part of the sugar mobilized in the liver fails to be utilized in the peripheral tissues. That blood sugar utilization is low during epinephrine hyperglycemia is suggested by the results obtained in some recent experiments on men (unpublished). Under postabsorptive conditions venous blood of a limb contains 4 to 8 mg. less sugar than arterial blood. When sugar is ingested (Foster (27)), or insulin injected (28), or most markedly, when sugar plus insulin is administered, the arteriovenous difference rises to 30 mg. and more, indicating that the peripheral tissues take up more blood sugar under these conditions. However, when hyperglycemia is produced by epinephrine injections, the arteriovenous difference remains unchanged, indicating that the peripheral tissues are unable to withdraw blood sugar at a higher rate.

DISCUSSION.

According to the generally accepted explanation epinephrine causes hyperglycemia, and after large doses, glycosuria, merely because it mobilizes liver glycogen. As pointed out in the introduction, hyperglycemia and glycosuria cannot be explained on this basis because there is not enough liver glycogen present to provide the tissues for hours with sugar at a rate which is greater than their normal ability to utilize sugar. This is illustrated by the following calculation. In the experiments in Table IV the average blood sugar was 174 mg. per cent 3 hours after the epinephrine injection. In order to produce a hyperglycemia of this magnitude in normal rats, glucose must be infused intravenously at a rate of 250 mg. per 100 gm. of body weight per hour (29). Hence, a mobilization of 750 mg. of liver glycogen or an initial glycogen content of the liver of 20 per cent is required, if the hyperglycemia of 174 mg. per cent is to be explained merely on the basis of glycogen mobilization in the liver. Actually there were only 218

mg. or 5.6 per cent liver glycogen present to start with (Table II), and the liver glycogen showed an increase after the epinephrine injection. This indicates that other factors play a rôle in the production of hyperglycemia besides mobilization of liver glycogen. In fact the experiments with epinephrine in Table IV, when taken at their face value, give no evidence that liver glycogen is being mobilized; only by means of a finer analysis could it be shown that mobilization of liver glycogen occurs.

Decreased utilization of blood sugar is one of the principal causes for the epinephrine hyperglycemia under postabsorptive conditions. The blood sugar rises after epinephrine injections, because the utilization of blood sugar in the peripheral tissues falls behind the supply of blood sugar by the liver, even if the supply is not much higher than normal. For this reason, amounts of sugar given off by the liver will cause hyperglycemia, amounts, which when injected into normal animals, have no effect whatever on the blood sugar level. Hitherto it has been assumed that a decreased utilization of blood sugar must be associated with a decreased carbohydrate oxidation. The present experiments reveal that after epinephrine injections carbohydrate oxidation is carried on at the expense of muscle glycogen and that the carbohydrate oxidation may be increased in spite of a diminished blood sugar utilization.[6]

The conversion of lactic acid derived from muscle glycogen into liver glycogen explains why epinephrine hyperglycemia can persist for several hours without causing a depletion of the glycogen stores of the liver. A decrease in liver glycogen is to be expected only if glycosuria develops. In rabbits during epinephrine glycosuria more sugar appears in the urine than can be accounted for by the disappearance of liver glycogen, indicating that resynthesis of liver glycogen from lactic acid occurs also in this species (30). It can now be understood why different observers obtained conflicting results when they determined the glycogen content of the liver after epinephrine injections. With small doses of epinephrine, or even with large doses when the glycogen content of the liver is low, resynthesis of liver glycogen from lactic acid exceeds mobilization, and consequently the liver glycogen is found higher after the injection. With large doses and a high liver gly-

[6] It is therefore not correct to speak of "adrenalin diabetes."

cogen the latter diminishes because the loss of sugar in the urine cannot entirely be compensated by resynthesis of liver glycogen.

It is surprising that an extrahepatic action of epinephrine on carbohydrate metabolism has not been emphasized before. The experiments on 24 hour fasting and on rats in the postabsorptive state indicate that the extrahepatic action of epinephrine consists in a mobilization of muscle glycogen. Whether this mobilization is responsible for the decreased utilization of blood sugar, or whether both processes are influenced independently by epinephrine, cannot be decided at present.

Since the end-product of glycogen breakdown in the muscle is lactic acid, epinephrine cannot raise the blood sugar level after removal of the liver. However, the influence of epinephrine on blood sugar utilization is demonstrable on hepatectomized animals, as will be shown in subsequent papers.

Insulin produces hypoglycemia because the supply of sugar falls behind blood sugar utilization, in spite of a rapid mobilization of liver glycogen. Epinephrine and insulin antagonize each other on account of their opposite effect on blood sugar utilization in the peripheral tissues.

SUMMARY.

1. A carbohydrate balance has been made on rats in the postabsorptive state by determining simultaneously sugar oxidation and disappearance of glycogen from the liver and the rest of the body. In the control rats 7.5 per cent more carbohydrate disappeared than was accounted for by oxidation; in the rats injected with epinephrine the agreement happened to be perfect; in the rats injected with insulin 13.4 per cent more carbohydrate was oxidized than could be accounted for by the disappearance of glycogen.

2. In the control rats 216 mg. of glycogen disappeared in 3 hours, 23 per cent being derived from the liver and 77 per cent from the rest of the body. In the animals injected with epinephrine all the glycogen which disappeared (298 mg.) came from the body, there being an actual increase in liver glycogen of 26 mg. After insulin 43 per cent of the disappearing glycogen (329 mg.) was derived from liver and 57 per cent from body glycogen.

3. In comparison with the control rats, the animals receiving

epinephrine showed an increase in carbohydrate oxidation of 19.5 per cent and the animals receiving insulin of 97.3 per cent. The blood sugar level in the three cases stood at 113, 174, and 69 mg. per cent respectively. Glycosuria was not produced by the dose of epinephrine injected (0.02 mg. per 100 gm. of rat).

4. The calories of the control and insulinized animals were within 0.5 per cent the same, indicating that there occurred an equicaloric replacement of carbohydrate oxidation for fat oxidation after the insulin injection. Epinephrine caused an increase in heat production of 24 per cent, 67 per cent of the extra calories being furnished by fat and 33 per cent by carbohydrate.

5. The mechanism of epinephrine action is discussed. After non-glycosuric doses the most prominent effect of epinephrine is observed in the peripheral tissues and consists in a mobilization of muscle glycogen and in a decreased utilization of blood sugar. Oxidation of muscle glycogen is increased under postabsorptive conditions, sharply distinguishing the action of epinephrine from the effects of pancreatectomy. Part of the lactic acid arising from the breakdown of muscle glycogen escapes removal by oxidation and is carried to the liver where it is deposited as glycogen. In this way a cycle of carbohydrates is established. Blood sugar derived from liver glycogen is utilized in the muscles and lactic acid derived from muscle glycogen is returned to the liver. Epinephrine hyperglycemia develops because the utilization of blood sugar is diminished in relation to the supply of blood sugar by the liver, even if the supply is not much higher than normal. Insulin is the hormone that leads to a preferential utilization of blood sugar and indirectly of liver glycogen. The antagonistic action between insulin and epinephrine is explained by their divergent effect on blood sugar utilization.

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THE MECHANISM OF EPINEPHRINE ACTION.

III. THE INFLUENCE OF EPINEPHRINE ON THE UTILIZATION OF ABSORBED GLUCOSE.

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The experiments in the preceding paper (1) were made on rats in the postabsorptive state. Under postabsorptive conditions blood sugar is being supplied by the liver, only a small amount of sugar is transported by the blood, glycogen synthesis is not taking place to any large extent, and carbohydrate metabolism is carried on mainly at the expense of preformed glycogen. These experimental conditions are favorable for observing the influence of epinephrine on glycogen utilization and it has been found that epinephrine injections cause a more rapid disappearance of muscle glycogen and an increase in liver glycogen in rats in the postabsorptive state. The present experiments on sugar-fed rats offer a different problem. During glucose absorption a large amount of sugar passes into the blood. This sugar must penetrate into the tissues prior to its utilization. In the tissues the sugar is disposed of, partly by oxidation, partly by conversion into glycogen, and carbohydrate metabolism is carried on mainly at the expense of the sugar that is circulating in the blood. These experimental conditions are favorable for observations on the influence of epinephrine on blood sugar utilization. The uptake of blood sugar was diminished in rats in the postabsorptive state following epinephrine injections. This effect of epinephrine should be more marked in sugar-fed rats, since during intestinal absorption 14 times more sugar is transported by the blood than under postabsorptive conditions. It was actually found that the same dose of epinephrine that produced only hyperglycemia in rats in the postabsorptive state, caused a marked glycosuria

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in sugar-fed rats. As will be shown later this glycosuria is mainly due to a decreased utilization of blood sugar in the peripheral tissues.

Methods.

In the present paper a comparison is made between the utilization of glucose in normal rats and in rats receiving epinephrine injections. The control series, consisting of ten individual experiments, has already been published in Table III of a previous paper (2), where all the necessary details are given. Both the control and the experiments with epinephrine were made within 2 months (March and April, 1927) and care was taken to spread them evenly over this period. In order to save space the control series is not reproduced again, with the exception of the average values which are given in Table III of the present paper. The technique used has also been described in the previous paper (2). In brief outline the experiments were performed in the following way. Male rats of 140 to 150 gm. of body weight were fasted for 22 hours. A metabolism fore period of 2 hours was made in a Haldane type metabolism apparatus. Enough glucose was then fed by stomach tube to permit the absorption to proceed for at least 5 hours, and the subcutaneous injection of epinephrine was made (0.02 mg. per 100 gm. of body weight). The respiratory metabolism was determined for 4 hours after the sugar feeding. The animals were then killed, and absorption and glycogen content of the liver and the rest of the body were determined in the usual manner. The glycogen values found after the 4 hours of glucose absorption were corrected for the preformed glycogen, 140 mg. being subtracted for the body and 7 mg. for the liver (see Table I of a previous paper (2)). The second metabolism period was made in a closed type of metabolism apparatus, which was used for numerous experiments on previous occasions, and which permits a determination of O_2 consumption by means of two independent methods. The average difference between the two O_2 determinations for the eight metabolism experiments here reported was 1.4 $\pm$ 0.8 per cent. Calculations of the amounts of sugar and fat oxidized were made from the non-protein R.Q. with the aid of the Zuntz-Schumburg-Lusk tables. The control rats were treated in exactly the same way except that no epinephrine was given.

Epinephrine injections have been shown to cause overventilation (3), which in turn may lead to the blowing off of CO_2 and consequently to R.Q.'s which are too high. After the period of overventilation the R.Q's may be found too low, owing to a retention of CO_2. The danger of erroneous R.Q.'s on account of disturbances in respiration is great in metabolism experiments of short duration. However, this danger is not met with in metabolism experiments extending over 4 hours, partly because the amount of CO_2 that could be blown off would be small in comparison to the amount of CO_2 arising from the metabolism during that time, partly because enough time elapses in which a disturbance of the acid-base equilibrium might be readjusted. Previous experiments (4) have shown that large doses of epinephrine cause an increase in the lactic acid content of the blood of rabbits and cats. In view of this, CO_2 determinations were made on the blood of control rats under the conditions obtaining at the beginning and end of the metabolism experiments with epinephrine. 4 hours after glucose feeding plus epinephrine, CO_2 values (for plasma saturated with alveolar air) of 51.4 to 56.7 volumes per cent were obtained. This in no way departs from the values found before epinephrine was injected. The acid-base balance was, therefore, the same at the beginning and end of the metabolism experiments with epinephrine, which is of importance for the interpretation of the R.Q.'s. Whether there was any change in plasma CO_2 at shorter time intervals after the injection was not ascertained.

Epinephrine Dose.

The dose used in the present work was the same as in the experiments on 24 hour fasting and rats in the postabsorptive state; namely, 0.02 mg. per 100 gm. of body weight, injected subcutaneously in 0.1 cc. of fluid. The dilution was prepared from the 1:1000 adrenalin chloride solution of Parke, Davis and Company. It is of importance to decide whether this dose is small or large in a physiological sense. The following points must be taken into consideration. There is good evidence that subcutaneously injected epinephrine is not absorbed at once into the blood stream. Epinephrine blocks its own passage into the circulation because of local vasoconstriction, and absorption can probably proceed only at the border of the anemic area. In this way a depot is

created in the subcutaneous tissue. Absorption from this depot can persist for several hours, because epinephrine is not rapidly destroyed in the subcutaneous tissue (Auer and Meltzer (5), Tatum (6), Luckhardt and Koppányi (7)). After the subcutaneous injection of 1 mg. of epinephrine per kilo, as a rule, no rise in blood pressure is observed in rabbits, cats, and dogs. Trendelenburg (8) concluded from this that the rate of absorption after such an injection must be below 0.0005 mg. of epinephrine per kilo per minute, because a constant intravenous infusion at this rate is just able to cause a rise in blood pressure of unnarcotized rabbits. Cannon and Rapport (9) found an average maximal rate of discharge of 0.003 mg. of epinephrine per kilo per minute from the adrenals of the cat. It appears that the rate at which subcutaneously injected epinephrine is absorbed into the blood stream is lower than the maximal rate of discharge of epinephrine from the adrenals. Trendelenburg (10) found in unanesthetized rabbits that glycosuria is produced at a rate of infusion of epinephrine which has no effect on blood pressure. Straub (11) and Tatum (6) have shown that glycosuria and hyperglycemia persist only as long as epinephrine enters the blood stream. This is mainly due to the fact that epinephrine is almost entirely destroyed upon one passage through the capillaries. Since in the present experiments the glycosuria of the rats persisted for 4 hours, epinephrine must have been absorbed for at least that length of time. The average sugar excretion from 0 to 1, from 1 to 2, from 2 to 3, and from 3 to 4 hours was 12, 13, 15, and 15 mg. respectively (Tables I and III). The investigation was not carried beyond 4 hours. The fact that the amounts of sugar excreted from hour to hour remained practically constant, suggests a rather constant rate of absorption of epinephrine from the subcutaneous tissue. Assuming that all the epinephrine injected, namely 0.02 mg. per 100 gm. of rat, was absorbed during the 4 hours, which it probably was not, one obtains a rate of absorption of $\frac{0.2}{240} = 0.0008$ per kilo per minute. This is lower than the average maximal output of the adrenals of the cat as determined by Cannon and Rapport. The rate of discharge of epinephrine from the adrenals of the rat is not known. The following figures are of interest in this connection. According to Herring (12) and Kuriyama (13) there are

0.034 to 0.043 mg. of epinephrine in the adrenals of a rat of 100 gm. of body weight, while in the present experiments at the most 0.02 mg. of epinephrine passed into the blood in the course of 4 hours. These points were discussed at some length in order to show that the rate at which epinephrine is absorbed from the subcutaneous tissue of the rat, when 0.02 mg. in 0.1 cc. of fluid is injected, falls within physiological limits. For this reason the results obtained with this dose might be expected to be of physiological significance.

Changes in Carbohydrate Metabolism 1, 2, and 3 Hours after Injection.

A carbohydrate balance was made for the 4 hour absorption period. It was of interest to investigate whether certain characteristic changes which are present 4 hours after the injection, can also be observed at earlier periods. Table I contains data on absorption, glycogen content of liver, and blood and urinary sugar of control and injected rats 1, 2, and 3 hours after glucose feeding. The respective values for the 4 hour absorption period may be found in Table II. It should be noted that the average weight of the rats in Table I was greater than that of the rats used for the 4 hour period. The highest average blood sugar (299 mg. per cent) was observed 1 hour after the injection. After 2 and 3 hours the blood sugar was lower (251 and 257 mg.), and a further fall in blood sugar occurred between the 3rd and 4th hour. In spite of this decrease in blood sugar the rate of sugar excretion in the urine remained practically constant from hour to hour.

The rats receiving epinephrine absorbed less glucose than the controls. This is noticeable after 2, 3, and 4 hours but not after 1 hour of absorption. An explanation for the slower rate of absorption cannot be given at present. It remains undecided whether in these experiments epinephrine had any inhibiting effect on intestinal motility, and furthermore, it is not known whether a decreased motility reduces the rate of absorption. Constriction of splanchnic blood vessels might also be considered. Neither of these explanations fits the fact that only 1 hour after the injection did the inhibition of absorption reach a marked degree. These observations suggest that an increased release of epinephrine from the adrenals may influence absorption.

Though the rats receiving epinephrine absorbed less glucose they

formed more liver glycogen than the control rats. With the exception of the 1st hour, the difference in glycogen deposition is significant as shown by the average deviation from the mean (Table I). The sugar balance for the 4 hour absorption period shows that the increased deposition of liver glycogen is not due to

TABLE I.

Influence of Epinephrine on Glucose Absorption, Glycogen Formation in Liver, and Sugar Excretion.

Average body weight 165.5 ± 10 gm. Values calculated per 100 gm. of body weight.

Length of absorption period.	Controls.			Epinephrine.			
	Glucose absorbed.	Glycogen formed in liver.	Blood sugar.	Glucose absorbed.	Glycogen formed in liver.	Blood sugar.	Urine sugar.
hrs.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
1	193	26		225	33	324	8
	259	28	137	182	43	267	13
	204	41	131	181	31	298	8
	213	36	126	229	27	307	18
2	217 ±21	33 ±6	131	204 ±23	34 ±5	299	12
	452	68	153	344	73	217	21
	318	55	159	318	97	321	21
	420	82	156	254	79	226	31
	370	68	144	270	81	241	27
3	390 ±46	68 ±7	153	296 ±33	83 ±7	251	25
	570	141	156	495	147	243	39
	564	156	161	543	185	251	25
	666	118	148	507	151	254	49
	627	101	149	498	172	261	67
	624	112	142	486	171	276	21
	609 ±35	126 ±18	151	507 ±16	165 ±13	257	40

an accelerating effect of epinephrine on the synthetic process. The actual mechanism involved will be discussed in the next section.

Sugar Balance after Epinephrine.

The individual experiments of the 4 hour period in Table II show that the results are uniform throughout. It will be sufficient

TABLE II.

Influence of Epinephrine on Sugar Oxidation and Glycogen Formation during 4 Hours of Glucose Absorption.

Average body weight 134.7 ± 3 gm. Values calculated per 100 gm. of body weight per 4 hours.

Fore period.		Absorption period.											
				Glycogen formed.			Glucose recovered.						
O_2	R.Q.	Glucose absorbed.	Sugar oxidized.	In liver.	In rest of body.	Total.			O_2	R.Q.	Urine N.	Urine sugar.	Blood sugar.
gm.		*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*per cent*	*gm.*		*mg.*	*gm.*	*mg.*
0.897	0.709	0.795	0.270	0.164	0.109	0.273	0.628	79.0	1.058	0.794	10.11	0.085	189
0.931	0.723	0.870	0.323	0.256	0.090	0.346	0.716	82.3	0.966	0.823	14.55	0.047	202
0.941	0.705	0.953	0.330	0.255	0.153	0.408	0.761	79.8	1.084	0.812	14.69	0.023	215
1.108	0.711	0.897	0.351	0.184	0.148	0.332	0.745	83.1	1.115	0.816	14.59	0.062	189
0.898	0.709	0.957	0.360	0.181	0.196	0.377	0.768	80.3	0.985	0.833	15.36	0.031	188
0.974	0.703	0.891	0.361	0.267	0.091	0.358	0.784	88.0	1.036	0.823	10.85	0.065	232
0.883	0.719	0.953	0.387	0.213	0.179	0.392	0.832	87.3	0.999	0.840	16.13	0.053	190
1.017	0.709	0.973	0.474	0.173	0.119	0.292	0.843	86.6	1.099	0.852	14.68	0.077	176

to consider the average values in Table III. The recovery of absorbed sugar amounts to 83.4 per cent in the experiments with epinephrine and to 86.5 per cent in the control experiments. The lower recovery in the former case is due to the fact that the blood

TABLE III.

Average Values Calculated from Table II.

The values for the controls are taken from a previous paper (2).

	Glucose.	Glucose plus epinephrine.
Fore period.		
O_2, *gm.*	0.954	0.956
R. Q.	0.715	0.712
Total calories	3.09	3.10
Glucose absorption period.		
Glucose absorbed, *gm.*	1.065	0.911
" oxidized "	0.465	0.357
Glycogen formed in liver, *gm.*	0.192	0.212
" " " rest of body, *gm.*	0.263	0.136
Total glycogen, *gm.*	0.455	0.348
Glucose recovered, *gm.*	0.919	0.760
" " *per cent.*	86.5	83.4
" lost, *gm.*	0.146	0.159
Glycogen formed / Sugar oxidized	1.00	0.97
Blood sugar, *mg.*	158	198
Urine " *gm.*		0.055
" N, *mg.*	15.65	13.87
O_2, *gm.*	0.885	1.043
R.Q.	0.884	0.824
Non-protein O_2, *gm.*	0.752	0.838
" R.Q.	0.900	0.828
Protein oxidized, *gm.*	0.096	0.085
Fat oxidized, *gm.*	0.090	0.190
Calories from protein	0.39	0.35
" " fat	0.85	1.79
" " sugar	1.74	1.33
Total calories	2.98	3.47

sugar is higher at the close of the experiment. Consequently more sugar is retained in the body fluids. Assuming that the blood sugar is in equilibrium with 50 per cent of the body weight, one accounts for an additional 20 mg. of sugar. The recovery in the experi-

ments with epinephrine is then 85.6 per cent which is close to that of the control experiments.

Owing to the difference in absorption of the control and injected rats, their sugar oxidation and glycogen formation cannot be compared directly. The respective values have to be calculated per 100 parts of absorbed sugar which eliminates the differences in absorption. This has been done in Table IV, with the data of Table III.

It will be noted in Table IV that the injected rats excreted 6 per cent of the absorbed glucose, while the control rats showed no glycosuria. The same dose of epinephrine, when injected into animals in the postabsorptive state, produced only hyperglycemia

TABLE IV.

Percentage of Sugar Oxidation and Glycogen Formation per 100 Parts of Absorbed Sugar.

Calculated from Table III.

	Parts per 100 parts of absorbed sugar.				
	Oxidised.	Deposited as liver glycogen.	Deposited as glycogen in rest of body.	Retained in body fluids.	Excreted in urine.
Controls	44	18	25	2.0	0
Epinephrine	39	23	15	5.0	6
Difference	−5	+5	−10	+3.0	+6

but no glycosuria (1). Yet, the rats in the postabsorptive state contained a large amount of liver glycogen (5.6 per cent) at the time of the injection while the rats in the present experiments contained practically no liver glycogen to start with. Nevertheless the sugar-fed rats excreted sugar in the 1st hour after the injection. The glycosuria of the sugar-fed rats is therefore related to the transportation of large amounts of sugar by the blood stream. In order to make clear how much sugar is transported in relation to the blood volume, the following example is offered. A rat of 100 gm. of body weight has a blood volume of approximately 7 cc., containing 7 mg. of sugar at a blood sugar level of 100 mg. per cent. In 4 hours 1065 mg. of glucose, or 152 times more sugar than is held by the blood, pass into it from the intestine. The

disposal of sugar in the tissues must therefore be very rapid and must be well adjusted to the rate of absorption in order to keep the blood sugar near the normal level. This is realized in the normal rat, where the blood sugar rises from 100 to 158 mg. per cent during an absorption period of 4 hours, corresponding to a retention of only 29 mg. of sugar in the body fluids out of the 1065 mg. of sugar which have passed into the blood. The rats receiving epinephrine, though they absorbed less sugar than the normal rats, showed a marked rise in blood sugar and in consequence of this, glycosuria. Obviously epinephrine must have interfered with the processes of sugar disposal in the tissues. Since the liver glycogen was higher after the epinephrine injection, the disturbance in sugar utilization must be sought for in the peripheral tissues.

Table IV indicates that the injected rats formed 10 parts less body glycogen than the control rats. It has not been ascertained whether this is due to an actual inhibition of glycogen synthesis in the muscles or whether part of the glycogen already deposited is mobilized again by epinephrine. The second alternative would be consistent with the mobilization of muscle glycogen which has been observed in 24 hour fasting rats and rats in the postabsorptive state after epinephrine injections. To the 10 parts of absorbed sugar which failed to be deposited as body glycogen must be added the 5 parts of absorbed sugar which are not oxidized (Table IV). This leaves a total of 15 parts of absorbed sugar which fail to be utilized in the peripheral tissues and which are therefore accumulating in the blood. 5 parts out of the 15 parts are stored in excess in the liver, 6 parts are excreted in the urine, and 3 parts are retained in the blood and body fluids, leaving 1 part unaccounted for. It is obvious that if the liver were able to store 15 parts in excess instead of only 5 parts, hyperglycemia and glycosuria would not develop. Either the liver is unable to store so large an excess of sugar or part of the glycogen deposited in excess is mobilized again by epinephrine. In both cases the liver plays only a secondary rôle in the production of glycosuria. The factor of primary importance is the excess of sugar accumulating in the blood on account of a decreased utilization of blood sugar in the peripheral tissues. The second factor is the inability of the liver to handle this excess of sugar, possibly because mobilization of

liver glycogen is taking place. This analysis shows that the glycosuria of the sugar-fed rats is mainly the result of an extrahepatic and not of a hepatic action of epinephrine. In this way the surprising observation that glycosuria develops during a period of increased glycogen storage in the liver finds a satisfactory explanation.

A factor which undoubtedly enters into the present experiments is an increased release of insulin from the pancreas, called forth by the large amounts of sugar passing through the blood. This insulin production enables the normal animal to utilize glucose at a rate of 2.5 gm. per kilo per hour for 4 hours without excreting sugar in the urine. It cannot be expected that under these conditions the small dose of epinephrine injected will entirely suppress the insulin action. The influence of larger doses of epinephrine has not been tried but it seems doubtful whether any dose will have this effect. It is of interest that during glucose absorption epinephrine injections have on every factor entering into the sugar balance an effect opposite to that of insulin injections. With insulin glycogen formation in the liver is decreased, with epinephrine increased. Oxidation and glycogen formation in the peripheral tissues are increased after insulin, decreased after epinephrine. Whether or not these two hormones act antagonistically by influencing the same process in an opposite direction must remain undecided as long as the actual point of attack on the carbohydrate molecule is unknown.

Influence of Epinephrine on Heat Production.

The following authors investigated the effect of epinephrine during sugar absorption. Wilenko (14) observed no rise in R.Q. in rabbits under urethane anesthesia after oral or subcutaneous administration of glucose plus epinephrine. He concluded that epinephrine decreases the ability of the body to oxidize sugar. This has been contradicted by Lusk and Riche (15), since they observed R.Q.'s of 0.9 in dogs after the ingestion of 50 gm. of glucose with simultaneous injection of large doses of epinephrine. The dogs were very restless and the authors therefore did not feel justified in drawing any conclusions as to the calorigenic action of epinephrine. In the present experiments with epinephrine the average R.Q. of the 4 hour glucose absorption period was 0.824 as

compared with the R.Q. of the fore period of 0.712. An increased heat production was observed in each experiment (Table II). The calories rose on an average from 3.1 in the fore period to 3.47 in the glucose absorption-epinephrine period, corresponding to an increase of 12 per cent (Table III). The control rats produced 3.09 calories in the fore period and 2.98 calories in the glucose absorption period, corresponding to a decrease of 3.5 per cent. During glucose absorption the injected rats produced 16.4 per cent more calories than the uninjected controls. In order to ascertain what foodstuff furnished the extra calories after the epinephrine injection, one has to compare the heat production of the glucose absorption period of the control rats with that of the injected rats. The control rats produced 0.85 fat and 1.74 carbohydrate calories, while the injected rats produced 1.79 fat and 1.33 carbohydrate calories. It is therefore evident that the increased heat production after epinephrine is entirely at the expense of fat oxidation. It seems significant that epinephrine is able to accelerate fat oxidation during a period of carbohydrate plethora, though the data so far available do not warrant the conclusion that there exists a direct relationship between the medullary hormone and fat metabolism.

SUMMARY.

1. Glucose was fed to 24 hour fasting rats and 0.02 mg. of epinephrine per 100 gm. of body weight was injected subcutaneously in 0.1 cc. of fluid. An estimate was made of the rate of absorption of epinephrine from the subcutaneous tissue after the injection of such a dose. Hyperglycemia and glycosuria were present after 1 hour and persisted for 4 hours after the injection. The sugar excretion in the urine remained practically constant from hour to hour. Absorption of glucose from the intestine was diminished. With the exception of the 1st hour, where there was no difference, the rats injected with epinephrine desposited more liver glycogen than the control rats.

2. A carbohydrate balance was made for the 4 hour absorption period. Per 100 parts of absorbed sugar, the rats injected with epinephrine deposited 10 parts less body glycogen and oxidized 5 parts less sugar than the control rats. This leaves 15 parts of absorbed sugar which failed to be utilized in the peripheral tissues. 5 parts out of the 15 parts were stored in excess in the liver, 6

parts were excreted in the urine, and 3 parts were retained in the body fluids. The observation that glycosuria develops during a period of increased glycogen deposition in the liver finds its explanation in a decreased utilization of blood sugar in the peripheral tissues.

3. During glucose absorption the rats injected with epinephrine produced 16.4 per cent more calories than the control rats, the extra heat being furnished exclusively by fat oxidation.

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